LEAKAGE OF PHOSPHATE IONS FROM GHOSTS OF RABBIT RED BLOOD CELLS(Dissertation_全文)

AUTHOR(S):

Utsumi, Hiroshi

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内海博司

LEAKAGE OF PHOSPHATE IONS FROM GHOSTS OF RABBIT RED BLOOD CELLS

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主論文 1.

CHARACTERISTICS OF THE LEAKAGE OF PHOSPHATE IONS FROM THE GHOSTS OF RABBIT RED BLOOD CELLS

HIROSHI UTSUMI

Laboratory of Radiation Biology, Faculty of Science,
Kyoto University, Kyoto (Japan)

SUMMARY

1. The mechanism of the leakage of phosphate ions from rabbit red cell ghosts was studied. Prelabelling of radioactive orthophosphate (^{32}P) was accomplished by reversible hypotonic hemolysis of intact cells in the presence of ^{32}P.

2. Three kinds of ghost preparations were used in this study. These reconstituted ghosts were different in their morphological properties, but not in their pattern of ^{32}P-leakage. Ghosts were more permeable to phosphate ions than were intact red cells.

3. Phosphate ions in ghosts were of three types; acid-insoluble, acid-soluble organic and acid-soluble inorganic phosphate. Only inorganic phosphate in ghosts was involved in the leakage of ^{32}P.

4. ^{32}P-leakage can be divided into at least two components (fast and slow), both of which obey first-order kinetics. The fast component is the fraction which is completely released by hypotonic rehemolysis.

5. The activation energies of both the fast and slow phase components of ^{32}P-leakage were calculated to be about 4,000 cal/mol.

6. Various inhibitors of glycolysis, active transport, or membrane SH-groups did not affect ^{32}P-leakage from ghosts.

7. It is suggested that the leakage of ^{32}P from ghosts is governed by the process of passive transport.

INTRODUCTION

The mechanism of cation transport on the red blood cells has been extensively studied by the use of reconstituted ghosts prepared by reversible hyponic hemolysis. One of the benefits of reconstituted ghosts is that reversible hemolysis allows them to be loaded with normally impermeable substances and various amounts of ions at the time of hemolysis[1-3]. The reconstituted ghosts, for example, give one of the best experimental proofs that

the machinery which governs ion distribution between the red cell and the external medium is located in the cell membrane[4-6]. In contrast to our knowledge about cation transport, we have as yet very little information about the permeability of reconstituted ghosts to anions. The study of the distribution of inorganic phosphate between the red cell and the suspending medium is complicated by the fact that inorganic phosphate ions enter the cells quickly and become bound with organic molecules to form phosphate esters[7-11]. Although phosphate uptake has been extensively investigated, phosphate leakage from the red cell has not been systematically examined. Even if phosphate uptake were clearly understood, it would still be necessary to study phosphate leakage independently because the basic mechanisms underlying inward and outward movements across the cell membrane could be different. It seems worthwhile to study the nature of phosphate leakage from red cell ghosts.

The present study led to the conclusion that the ghosts are more permeable to phosphate ions than intact red cells and that this leakage process can be divided into two components both of which were non-metabolic in nature.

MATERIALS AND METHODS

Blood from a normal healthy rabbit was placed in a test tube containing sodium heparin at a final concentration of 10 units/ml of blood. Immediately after bleeding, blood was centrifuged at 15,000xG for 5 minutes. The packed red blood cells were treated as follows:

Preparation of "Mg-ghosts" and "ATP-Mg-ghosts": One volume of packed cells ($1x10^{10}$ cells/ml) was added to 10 volumes of ice cold hemolyzing solution (pH 7.5 adjusted by 0.1 N NaOH) containing carrier-free radioactive orthophosphate (12 μCi/ml ^{32}P) and either 4 mM $MgCl_2$ (Mg-ghosts) or 4 mM ATP-disodium salt and 4 mM $MgCl_2$ (ATP-Mg-ghosts). Within 2-3 minutes, 3 M NaCl solution was quickly added to give a final concentration of about 150 mM. The hemolysate was incubated for 20 minutes with shaking in a water bath at 37° C in order to insure the process of reconstitution[4-6]. The hemolysate was then centrifuged at 20,000xG at 0° C for 15 minutes and the reconstituted sedimented ghosts were washed 4 times with buffered salt solution consisting of 50 mM NaCl, 10 mM KCl and 90 mM tris-HCl (pH 7.5).

Preparation of "Common ghosts (C-ghosts)": One volume of packed cells ($1x10^{10}$ cells/ml) was hemolyzed

by 10 volumes of ice cold distilled water containing ^{32}P (12 μCi/ml) which had been adjusted to pH 7.5 with 0.1 N NaOH. After standing for 20 minutes at room temperature, the hemolysate was centrifuged 20,000xG at 0° C for 15 minutes and the sedimented ghosts were washed 4 times by centrifugation with a solution consisting of 9 parts of 12 mM $MgCl_2$ and 1 part of 17 mM tris-HCl (pH 7.5).

An important difference between the above two ghost preparations is that the process of reconstitution of C-ghosts occurred when they were incubated in the isotonic buffered salt solution, i.e., at the start of the experiments, while that of Mg-ghosts or ATP-Mg-ghosts had already been accomplished before the start of the experiments. The morphological properties of the ghost preparations obtained by these three procedures were determined by phase contrast microscopy.

Time course experiments: After the final washing, one volume of packed ghosts was resuspended in 30 volumes of ice cold buffered salt solution. After removal of a zero time sample, parts of the suspensions were incubated for up to 3 hours, with constant shaking, in a water bath at 37° C. The start of incubation was regarded as zero time of incubation. At appropriate intervals during the incubation, 3 ml aliquots were withdrawn and immediately centrifuged at 20,000xG at 0° C for 15 minutes. One ml of

the supernatant was pipetted into a stainless steel planchet with a diameter of 2.5 cm. The test samples in the planchets were dried under an infrared lamp, and the radioactivity was measured by a GM-counter (ALOKA Co., Ltd., Model TDC-2).

The amount of ^{32}P in the unit volume of the over-all suspension mixture was also measured. The total amount of ^{32}P retained in ghosts was obtained as the ^{32}P count in the over-all suspension mixture minus that in the supernatant. The total amount of ^{32}P-leakage from ghosts during incubation was obtained by subtracting the radioactivity in the supernatant at the start of incubation from the corresponding value found at a given incubation period.

The sedimented ghosts, as well as the supernatant, were treated with ice cold TCA [trichloroacetic acid; final concentration of 5 % (W/V)] and centrifuged. The radioactivity in an aliquot of the supernatant, i.e., the acid-soluble phosphate fraction, was measured by the method described above. The inorganic phosphate (Pi) in the acid-soluble phosphate fraction was converted into phospho-molybdate and extracted with an isobutanol-benzene (1:1) mixture by a modification of the method of Martin and

Doty[12], and the radioactivity of the extract was counted in the same manner. The amount of ^{32}P in the acid-soluble organic phosphate (designated as Po) was calculated as the difference between the amount of ^{32}P in the acid-soluble fraction and that in the Pi fraction. The amount of ^{32}P in the acid-insoluble phosphate fraction was calculated as the difference between the total amount of ^{32}P and that in the acid-soluble fraction. The amount of ^{32}P in various fractions was expressed as a percentage of the total amount of ^{32}P in the ghosts at the start of incubation.

Hypotonic rehemolysis experiments: The procedure was similar to that of the time course experiments. At various intervals after incubation, aliquots of the ghost suspension were withdrawn and divided into two equal parts. One was used for measuring the radioactivity in the acid-soluble fraction of ghosts. The other was rehemolyzed with distilled water (1:30) and the resultant hemolysate was centrifuged after about one hour. Both the supernatant and the sediment were treated with 5 % TCA solution for measuring the radioactivity in acid-soluble phosphate and Pi fraction. The percentage of ^{32}P released from the ghosts by the hypotonic treatment was estimated from the radioactivity in the supernatant.

Temperature dependence experiments: The time course of the leakage of ^{32}P from ghosts was investigated at temperatures ranging from 0° to 39° C by the use of a temperature-regulated water bath.

Chemicals: ^{32}P was from Radiochemical Centre, England, distributed by the Japan Radioisotope Assoc. Disodium ATP and ouabain were purchased from Sigma Chemical Co.

RESULTS

When ghosts were prepared in the hemolyzing solution containing ^{32}P, about 75 % of the radioactivity in the original solution was found in the ghosts. Part of the ^{32}P originally distributed in the ghosts was removed by washing, but about 45 % of it was retained even after 4 washings. The yield of ghosts was between 60 and 80 % of the volume of packed red cells; this was also confirmed by cell counts in a hemocytometer.

The morphological properties of three kinds of ghosts were examined by phase contrast microscopy. ATP-Mg-ghosts consisted of a rather homogeneous population, mostly biconcave discs, although a few crenated discs were usually seen among them. C-ghosts were most heterogeneous in shape and size. Electron microscopy revealed that C-ghosts

consisted of two cell types: one in the form of biconcave discs with much more cellular content than ATP-Mg-ghosts; the other with little intracellular substance and usually irregular in shape. Mg-ghosts were intermediate in their morphological properties between C-ghosts and ATP-Mg-ghosts The appearance of the ghosts became somewhat worse during the course of the subsequent incubation for 3 hours.

A fair amount of ^{32}P was found in the supernatant at the start of incubation, and this amount varied slightly with each preparation. Therefore, the total amount of ^{32}P-leakage from the ghosts was obtained by subtracting this value from the observed value. The time course of the leakage of ^{32}P from ATP-Mg-ghosts incubated in buffered salt solution is shown in Fig. 1. The leakage of ^{32}P was fast in the first phase and slow in the second phase. The leakage of ^{32}P in the form of acid-insoluble or acid-soluble organic ^{32}P was negligible. Therefore, the total leakage of ^{32}P was attributed to the leakage of ^{32}P in the inorganic form. Experiments on Mg-ghosts and C-ghosts gave similar results; i.e., the morphological differences did not influence the leakage of ^{32}P.

In order to determine the primary source of ^{32}P-leakage, the following quantities were measured at various

intervals: the amount of total ^{32}P, the inorganic phosphate (Pi) and the acid-soluble ^{32}P leaking from, and that remaining in, ghosts. The difference between the amounts of acid-soluble ^{32}P and inorganic ^{32}P represented the acid-soluble organic phosphate (Po). Fig. 2 shows that acid-insoluble ^{32}P and ^{32}Po pools in ATP-Mg-ghosts remained relatively constant. The acid-soluble ^{32}P in ghosts decreased markedly with incubation and there was an inverse quantitative relationship between the amount of ^{32}P leaking from ghosts and that of the acid-soluble ^{32}P remaining in them. The results indicate that ^{32}P-leakage is derived from the acid-soluble ^{32}P fraction in ghosts, especially from the Pi fraction. Experiments on Mg-ghosts and C-ghosts gave similar results.

Very diverse values were obtained for the amount of acid-soluble ^{32}P in ghosts at the start of incubation ($\underline{x}$). Fig. 3 shows that this variation can scarcely depend on the differences of ghost preparation, and that $\underline{x}$ has a roughly linear correlation with the amount of ^{32}P leaking from the ghosts during the first hour of incubation ($\underline{y}_1$). Therefore, the difference due to ghost preparation was neglected in the calculation of the regression equation ($\underline{y}_1$) and the correlation coefficient (ρ) shown in Fig. 3.

In order to analyze the ^{32}P-leakage process, the logarithm of the per cent of ^{32}P remaining in ghosts ($\underline{w}$) was plotted against the incubation time ($\underline{t}$) as shown in Fig. 4 (curve (A)). The amount of ^{32}P remaining in the ghosts ($\underline{w}$) was the total amount of ^{32}P in ghosts at the start of incubation (100) minus the total amount of ^{32}P-leakage during incubation ($\underline{y}$). Curve (A) shows that ^{32}P remaining in ghosts was lost more rapidly during the first 40 minutes than during the subsequent incubation. Two or more simultaneous processes were suggested to account for the ^{32}P-leakage from ghosts. It can be assumed that after long enough incubation, the second part of the curve may run parallel with the abscissa because of the un-releaseable compartment of ^{32}P (acid-insoluble ^{32}P fraction) in ghosts. When the value of acid-insoluble ^{32}P was subtracted from each experimental value and the results were plotted against incubation time, curve (B) was obtained. Since the second part of curve (B) appears straight, it was extrapolated back to zero time and the extrapolated values were subtracted from curve (B). The resultant plotting also gave a straight line ("backward projection" technique). This means that curve (B) can be resolved into two logarithmic functions and represented by the following equation ($\underline{w}_B$):

$$W_B = \Delta_1 e^{-k_1 t} + \Delta_2 e^{-k_2 t} \quad , \quad \Delta_1 + \Delta_2 = x \qquad (1)$$

where Δ is the size of the ^{32}P compartment in ghosts (given as a percentage of total radioactivity); k is the rate constant of ^{32}P-leakage from ghosts; t is the incubation time and the subscripts 1 and 2 refer to the fast and the slow phase of ^{32}P-leakage, respectively; x is the amount of acid-soluble ^{32}P in ghosts at the start of incubation.

Therefore, curve (A) can be represented by the following equation (w_A), which is the amount of ^{32}P remaining in ghosts at incubation (t):

$$W_A = \Delta_1 e^{-k_1 t} + \Delta_2 e^{-k_2 t} + \Delta_3 e^{-k_3 t}$$
$$\Delta_1 + \Delta_2 + \Delta_3 = 100 \qquad (2)$$

where Δ_3 is the size of the acid-insoluble ^{32}P compartment in ghosts, the rate constant k_3 will be zero.

The ^{32}P-leakage from ghosts at incubation time (t) can be represented by the following equation (y):

$$y = \Delta_1 (1 - e^{-k_1 t}) + \Delta_2 (1 - e^{-k_2 t})$$
$$\Delta_1 + \Delta_2 = x \quad (= 100 - \Delta_3) \qquad (3)$$

The mean values of Δ_1, Δ_2, Δ_3, k_1, k_2 and k_3 obtained from 16 experiments are shown in table I.

When one (hour) for t and the values given in table I for Δ and k were substituted in equation (2), the amount

of ^{32}P-leakage for the first hour of incubation was 23.9 %. On the other hand, the equivalent value was found to be 22.9 % on substituting the sum of Δ_1 and Δ_2 in table I for $\underline{x}$ into regression equation ($\underline{y}_1$) in Fig. 3, even though regression equation ($\underline{y}_1$) and equation (2) were derived from different experimental data.

When the ghosts withdrawn at the start of incubation were subjected to hypotonic treatment, the amount of ^{32}P released from them by rehemolysis ($\underline{z}$) was correlated linearly with that of the acid-soluble ^{32}P in the ghosts before rehemolysis ($\underline{x}$) (Fig. 5). The regression equation ($\underline{z}$) and correlation coefficient (ρ) are shown in Fig. 5. Substitution of $\underline{x}$ from regression equation ($\underline{y}_1$) into regression equation ($\underline{z}$) gives $\underline{y}_1 = 1.03\underline{z} + 1.1 \fallingdotseq \underline{z}$. This equation indicates that the amount of ^{32}P released by rehemolysis is approximately equal to the amount of ^{32}P leaking during the first hour of incubation.

Fig. 6 shows the results of the time-course rehemolysis experiment. The curve of the acid-soluble ^{32}P remaining in ghosts before rehemolysis (I), as well as that of the ^{32}P released by rehemolysis (II), is represented as the semi-logarithmic plot against incubation time. Curve (I) is equivalent to curve (B) in Fig. 4 and can be

be decomposed into two components, a fast and a slow phase. Curve (II) shows a remarkable decrease according to the time during which the ghosts were incubated before hypotonic treatment. Curve (II) was also analyzed into two components. The greatest similarity was between the fast phase component of curve (I) and that of curve (II). From this analysis, it may be concluded that the fast phase component of acid-soluble ^{32}P, that is, the leaky component of ^{32}P-leakage, was predominantly released from ghosts by hypotonic treatment. On the other hand, it is shown by equation (2) or regression equation ($\underline{y}_1$) that 96.7 % of the fast phase component leaks during the first hour of incubation. These findings seem to indicate that regression equation ($\underline{y}_1$) is approximately equal to regression equation ($\underline{z}$).

To test the effect of temperature on the ^{32}P-leakage process characterized by $\underline{\lambda}$ and $\underline{k}$, kinetic experiments were carried out at 7 different temperatures ranging from 0° to 39° C. The $\underline{k}$ and the $\underline{\lambda}$ at each temperature shown in Figs. 7 and 8 were determined by the same method used to obtain equation (1) or (2). The λ_1 was strongly dependent on temperature and became zero at about 0° C. On the other hand, the λ_3 (amount of acid-insoluble ^{32}P) was independent

of it and the λ_2 increased according to the relation written in the equation ($\lambda_1 + \lambda_2 = x$). The results show that the fast phase component of the ^{32}P-leakage disappeared near 0° C and only the slow phase component remained. On the basis of these results, the marked variation of x shown in Fig. 3 would be explained by the differences of temperature in the process of washing, which was usually performed at room temperature (5° - 33° C) and sometimes at 0° C. The higher the temperature, the more of the leaky acid-soluble ^{32}P fraction in ghosts may be lost during washing.

The Arrhenius equation relating the rate constant (k) and the absolute temperature (T) is $d \ln k / d(1/T) = -E/R$, where R is the gas constant (1.987 calories per deg. per mol.) and E is the activation energy. The logarithm of the rate constant of ^{32}P-leakage (k) changed linearly with the reciprocal of the absolute temperature of incubation (T) (Fig. 8). Therefore, the energy of activation (E) was calculated from the Arrhenius equation by plotting log k against $1/T$, where the slope of the line is equal to $-0.219\ E$. Both of the activation energies for k_1 and k_2 were found to be 4,000 calories per mole. This suggests that the leakage of ^{32}P from ghosts was by simple diffusion.

In order to examine further the nature of the ^{32}P-leakage, the effect of various drugs on ^{32}P-leakage was tested. Sodium fluoride (10^{-3} M) and iodoacetic acid (10^{-3} M, metabolic inhibitors of glycolysis) had no effect on ^{32}P-leakage. The fluoride has been found to affect also passive cation permeability[13]. However, the above results reveal that this mechanism had no relation to ^{32}P-leakage. As might be expected, dinitrophenol (10^{-4} M, inhibitor of oxydative phosphorylation) did not change the leakage. The inhibition of membrane SH-groups by p-chloromercury benzoate (10^{-4} M) and n-etylmaleimide (10^{-3} M) did not alter ^{32}P-leakage. Ouabain (10^{-8}- 10^{-4} M, inhibitor of active cation transport[14]) also had no effect. This indicates that the active cation transport mechanism has no relation to ^{32}P-leakage.

DISCUSSION

Kashket et al.[15] reported that while the amount of inorganic phosphate in red cells increased during prolonged storage, the leakage from cells was negligible. Our study of the phosphate leakage from prelabelled intact red cells incubated in the same way as the ghosts showed that the total phosphate leakage was under one per cent even after

2 hours of incubation. The data on the leakage of ^{32}P from ghosts indicate that they are more permeable to phosphate ions than are intact red cells. However, it should be mentioned that the ghosts used in this study preserved normal cation permeability and could be used in the studies of active and passive transport[5,6].

There is a possibility that unknown defects of membrane function were caused by hemolysis and that the defects of cation permeability, but not of anion permeability, were repaired during the subsequent preparation. The recovery of cation permeability of ghosts has been found to depend on the temperature or on the presence of alkaline earth ions at the time of hemolysis[16, 17], neither of which, however, had any measurable effect on ^{32}P-leakage.

The incorporation of ^{24}Na and ^{42}K cations into red cell ghosts has been studied by Hoffman[5] under the same hemolytic conditions. Both ^{24}Na and ^{42}K reached an isotopic equilibrium between the ghosts and the hemolyzing solution and, after the subsequent 4 washings, the amount of ^{24}Na and ^{42}K in the ghosts was reduced to 22.9 and 13.7 %, respectively.

In contrast to the cations, the amount of ^{32}P

incorporated into the ghosts was 3 to 5 times that in the hemolyzing solution, and the intracellular radioactivity after 4 washings was about 45 % of the initial activity. This suggests the existence of binding sites of phosphate ions. The fact that only a fraction of ^{32}P was released from the ghosts by hypotonic treatment strongly supports the above conclusion. These findings are strengthened by the results of Agren et al.[18] that the capacity of ghosts for phosphate ion incorporation was very large. In our experiments, the ^{32}P leaking from ghosts was inorganic phosphate, and a part of it not only leaked slowly from the ghosts during incubation but also was released in minimal amounts by hypotonic treatment. This suggests that inorganic phosphate ions were loosely bound with some membrane constituents.

The kinetics of ^{32}P-leakage is a first order process similar to that of sodium ions[19]. There is a possibility that phosphate ions are exchanged for a number of unknown minor compartments consisting of the various phosphate esters in ghosts, but such exchanges could not be detected since the acid-soluble organic phosphate and the acid-insoluble phosphate in ghosts were constant in amount during the incubation.

An explanation of two-phase leakage curves is that

there may be a release of phosphate ions from the membrane surface of ghosts. However, this seems doubtful since even several washings before incubation could not significantly affect ^{32}P-leakage and the fast phase components of ^{32}P-leakage were derived not from the cell surface, but from the intracellular compartments as suggested by hypotonic rehemolysis experiments (Figs. 5 and 6). An alternative explanation is that there may be two or more intracellular compartments in ghosts from which phosphate ions leak at different rates. These compartments can be presumed on the basis of the difference in affinity for various cations of membrane constituents. The component which shows slow leakage may correspond to the phosphate ion leakage in intact red cells, and the component which leaks fast may be characteristic of ghosts. A complete understanding of the nature of two phase leakage must await further experimentation, and it should be pointed out that these calculations were subject to all the uncertainties of the "backward projection" technique as described by Van Liew[20].

Glader and Omachi[19] reported that phosphate efflux in human red cells had a high apparent energy of activation. The activation energy of phosphate leakage from ghosts of rabbit red cells, however, was 4,000

calories per mole when calculate by the Arrhenius equation. The present data suggest the non-metabolic nature of phosphate transfer from ghosts to medium, as a similar value would be expected if diffusion was the major factor governing the leakage of inorganic phosphate. The non-metabolic nature of the leakage process will be further substantiated by the effect of various drugs on phosphate leakage.

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LEGENDS TO FIGURES

Fig. 1. The time course of ^{32}P-leakage from ATP-Mg-ghosts incubated in buffered salt solution: Total leaking ^{32}P (o), leaking acid-soluble ^{32}P (o), leaking ^{32}Pi (Δ). Each curve represents the percentage of the total amount of ^{32}P in ghosts at the start of incubation.

Fig. 2. The change in distribution of ^{32}P in and outside ATP-Mg-ghosts during incubation in buffered salt solution: The amount of ^{32}P in the acid-soluble organic phosphate (Po) was calculated as the difference between the amount of ^{32}P in the acid-soluble fraction and that in the Pi fraction. Each curve represents the percentage of the total amount of ^{32}P in ghosts at the start of incubation (acid-insoluble ^{32}P + acid-soluble ^{32}P + leaking ^{32}P = 100).

Fig. 3. The relation of the amount of ^{32}P leaking from ghosts during the first hour of incubation ($\underline{y}_1$) to that of acid-soluble ^{32}P in ghosts at the start of incubation ($\underline{x}$): ATP-Mg-ghosts (c), Mg-ghosts (Φ), C-ghosts (o).

The line is represented by the regression equation shown. See text for further explanation.

Fig. 4. Semi-logarithmic plot of the percentage of ^{32}P remaining in ghosts ($\underline{w}$) against the incubation time ($\underline{t}$) calculated from data in Fig. 1: The amount of ^{32}P remaining in ghosts ($\underline{w}$) was obtained as the total amount of ^{32}P in ghosts at the start of incubation (100) minus the total amount of ^{32}P leakage from the ghosts during incubation ($\underline{y}$). Curves (A) and (B) can be represented by $W_A = \lambda_1 e^{-k_1 t} + \lambda_2 e^{-k_2 t} + \lambda_3 e^{-k_3 t}$, $\lambda_1 + \lambda_2 + \lambda_3 = 100$ and $W_B = \lambda_1 e^{-k_1 t} + \lambda_2 e^{-k_2 t}$, $\lambda_1 + \lambda_2 = \alpha\ (= 100 - \lambda_3)$, respectively, where $\underline{x}$ is the amount of acid-soluble ^{32}P in the ghosts at the start of incubation. The curves were analyzed by the "backward projection" technique. See text for further explanation.

Fig. 5. Relationship between the amount of acid-soluble ^{32}P in ghosts before ($\underline{x}$) and that of ^{32}P released from ghosts after hypotonic treatment ($\underline{z}$): ATP-Mg-ghosts (○), Mg-ghosts (⊕), C-ghosts (○). The ghosts withdrawn at the start of incubation were used in this experiment. The line is represented by the regression equation shown.

Fig. 6. Semilogrithmic plot of the percentage of ^{32}P released from ATP-Mg-ghosts by hypotonic treatment: Curves (I) and (II) represent the amount of acid-soluble ^{32}P remaining in ghosts before (○) and that of ^{32}P released from ghosts after hypotonic treatment (▲), respectively. The numbers on the abscissa indicate the length of time the ghosts were incubated before treatment. Curves (I) and (II) were also analyzed by the method used to obtain equation (I). See text for further explanation.

Fig. 7. Effect of temperature on size of ^{32}P compartment in the ghosts (λ) of equation (1): Kinetic experiments were carried out 7 different temperatures ranging from 0° to 39° C. The λ at each temperature was determined by the method used to obtain equation (1).

Fig. 8. Common logarithm of the rate constant ($\underline{k}$) expressed as function of the reciprocal of the absolute temperature ($\underline{T}$): The energy of activation ($\underline{E}$) was calculated from the Arrhenius equation and from the slopes of the lines. The activation energy for both $\underline{k}_1$ and $\underline{k}_2$ was 4,000 calories per mole.

TABLE I

Rate constant of ^{32}P-leakage ($\underline{k}$) and size of ^{32}P compartment ($\underline{\lambda}$) of equation (1) or (2)

	Fast phase component	Slow phase component	Constant phase component
Rate constant ($hour^{-1}$)	$\underline{k}_1 = 3.407 \pm 0.236$	$\underline{k}_2 = 0.134 \pm 0.007$	$\underline{k}_3 = 0$
Size of ^{32}P compartment(%)	$\underline{\lambda}_1 = 20.3 \pm 2.1$	$\underline{\lambda}_2 = 27.0 \pm 3.4$	$\underline{\lambda}_3 = 52.7 \pm 4.0$

The time course curve of ^{32}P-leakage in each experiment was analyzed by the "backward projection" technique into three components, the $\underline{\lambda}$ and $\underline{k}$ of these three components are expressed as the mean values and standard error of the mean of 16 separate experiments. The differences of preparation of the ghosts was neglected in the calculation. See text for further explanation.

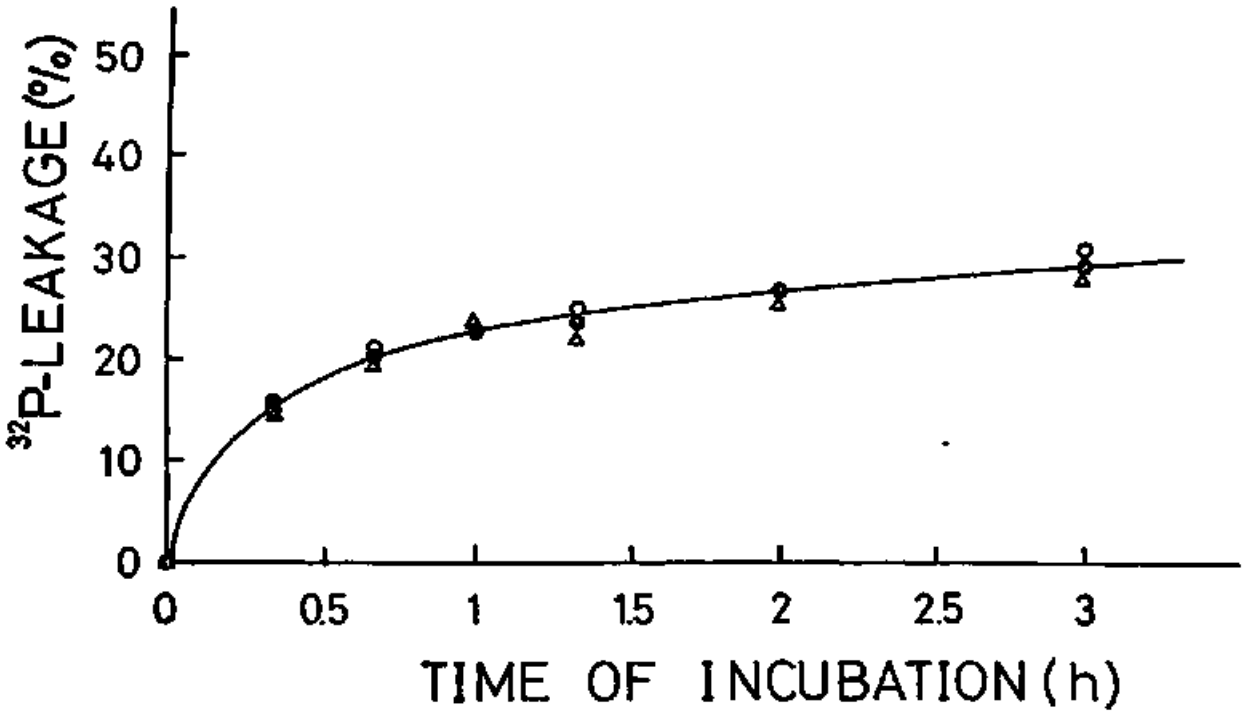


Fig. 1.

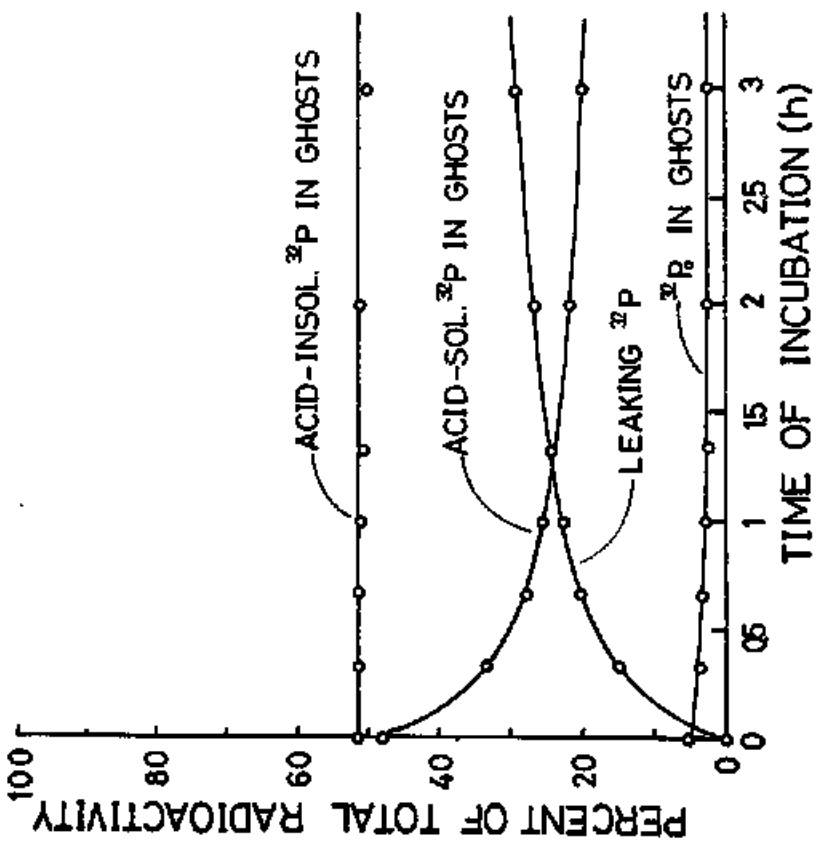

PERCENT OF TOTAL RADIOACTIVITY
100
80
60
40
20
0
0
0.5
1
1.5
2
2.5
3
TIME OF INCUBATION (h)
ACID-INSOL. ^{32}P IN GHOSTS
ACID-SOL. ^{32}P IN GHOSTS
LEAKING ^{32}P
$^{32}P_i$ IN GHOSTS

Fig. 2.

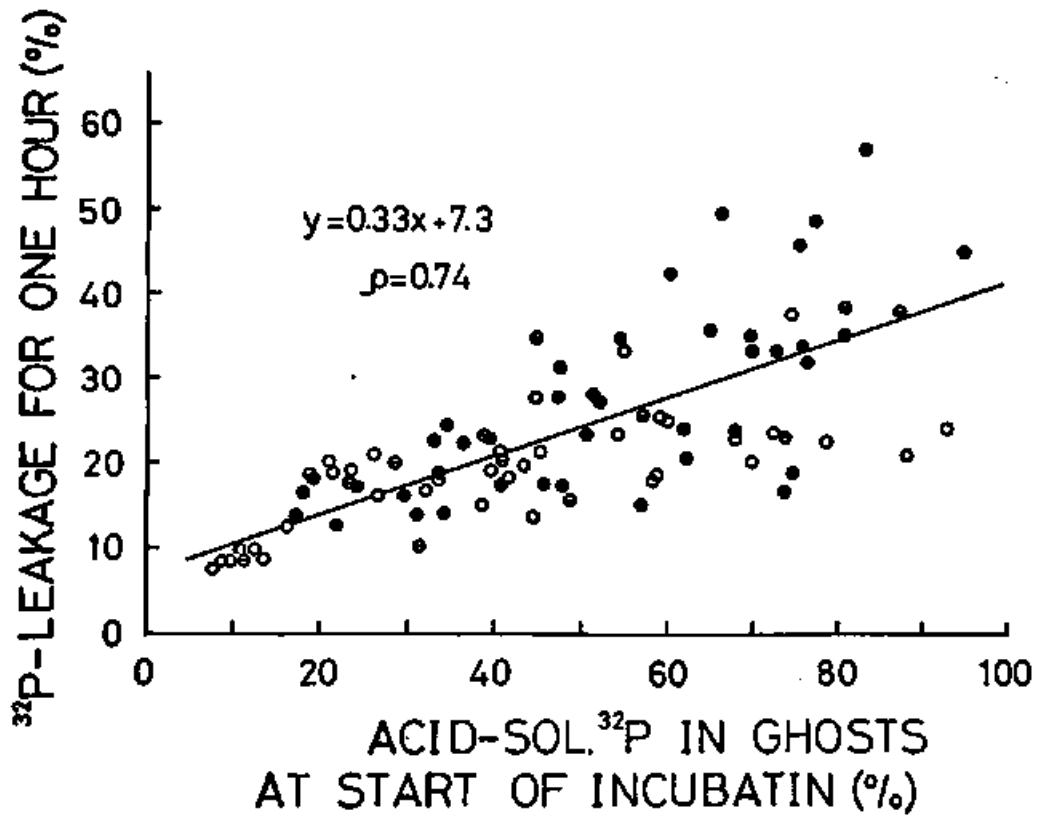

^{32}P-LEAKAGE FOR ONE HOUR (%)
y=0.33x+7.3
ρ=0.74
0
10
20
30
40
50
60
0
20
40
60
80
100
ACID-SOL.^{32}P IN GHOSTS
AT START OF INCUBATIN (%)

Fig. 3

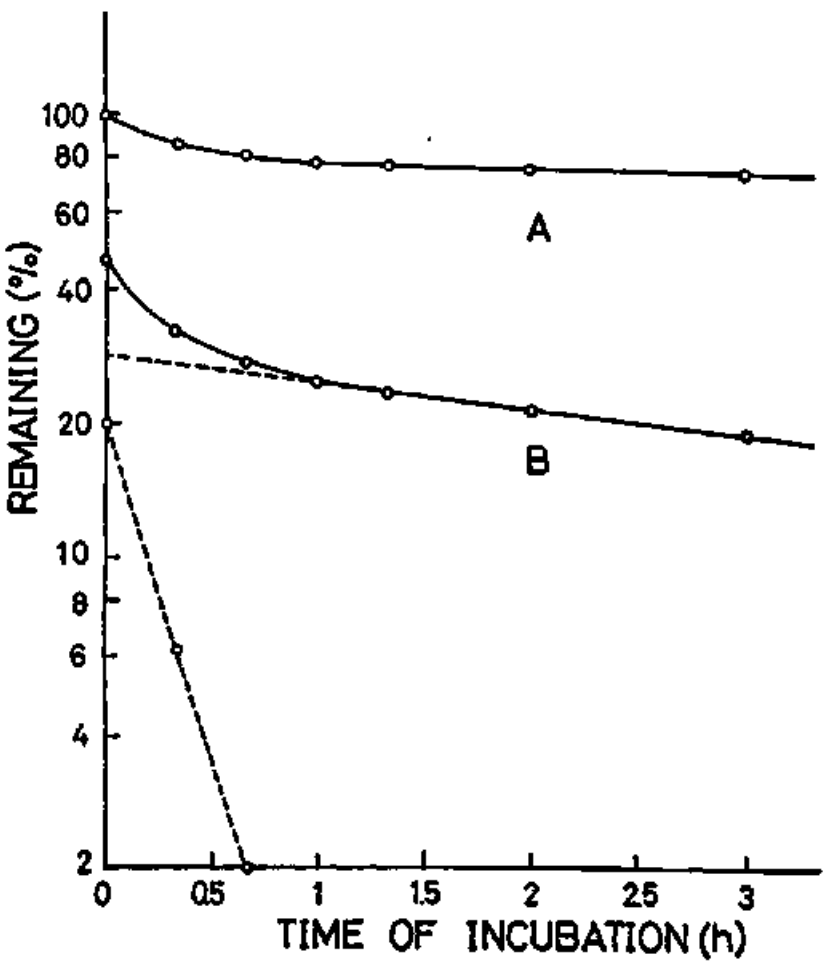

100
80
60
40
20
10
8
6
4
2
REMAINING (%)
A
B
0
0.5
1
1.5
2
2.5
3
TIME OF INCUBATION (h)

Fig. 4.

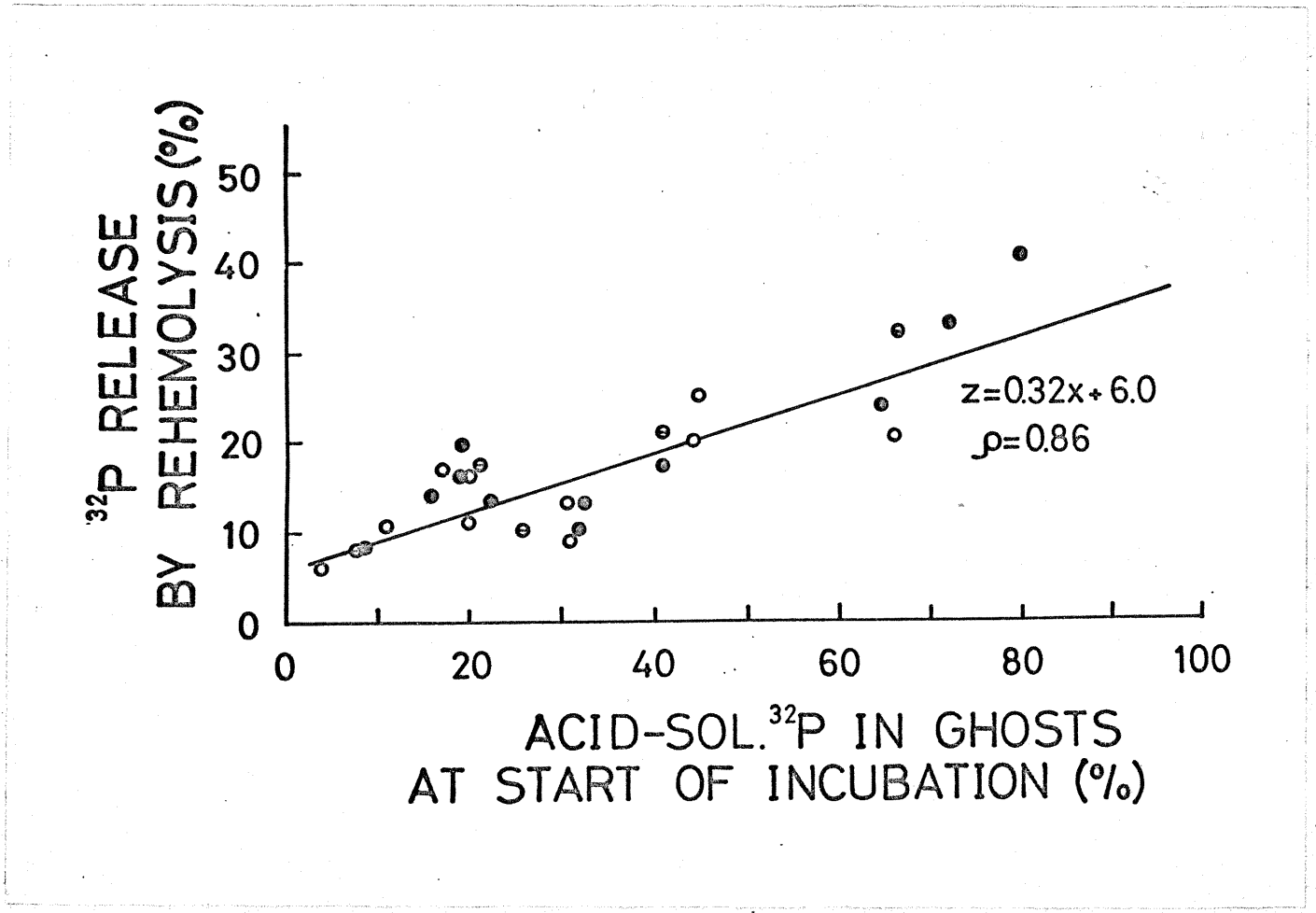

^{32}P RELEASE BY REHEMOLYSIS (%)
50
40
30
20
10
0
0
20
40
60
80
100
ACID-SOL. ^{32}P IN GHOSTS AT START OF INCUBATION (%)
z=0.32x+6.0
ρ=0.86

Fig. 5

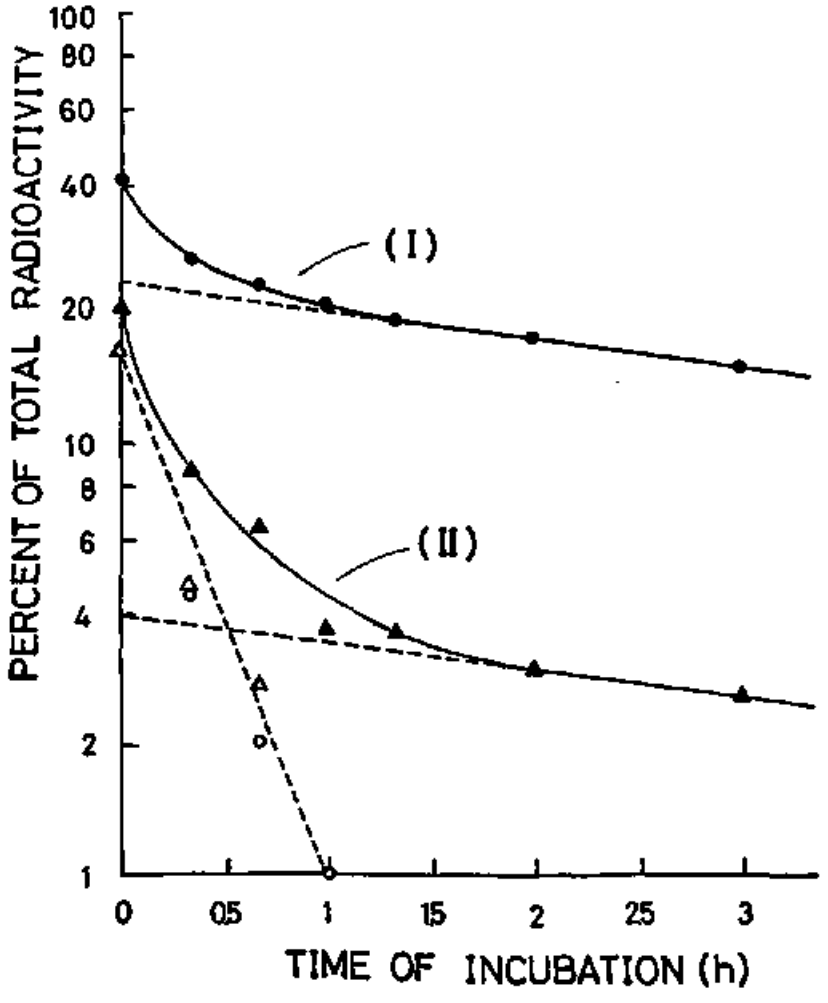

PERCENT OF TOTAL RADIOACTIVITY
100
80
60
40
20
10
8
6
4
2
1
(I)
(II)
0
05
1
15
2
25
3
TIME OF INCUBATION (h)

Fig. 6.

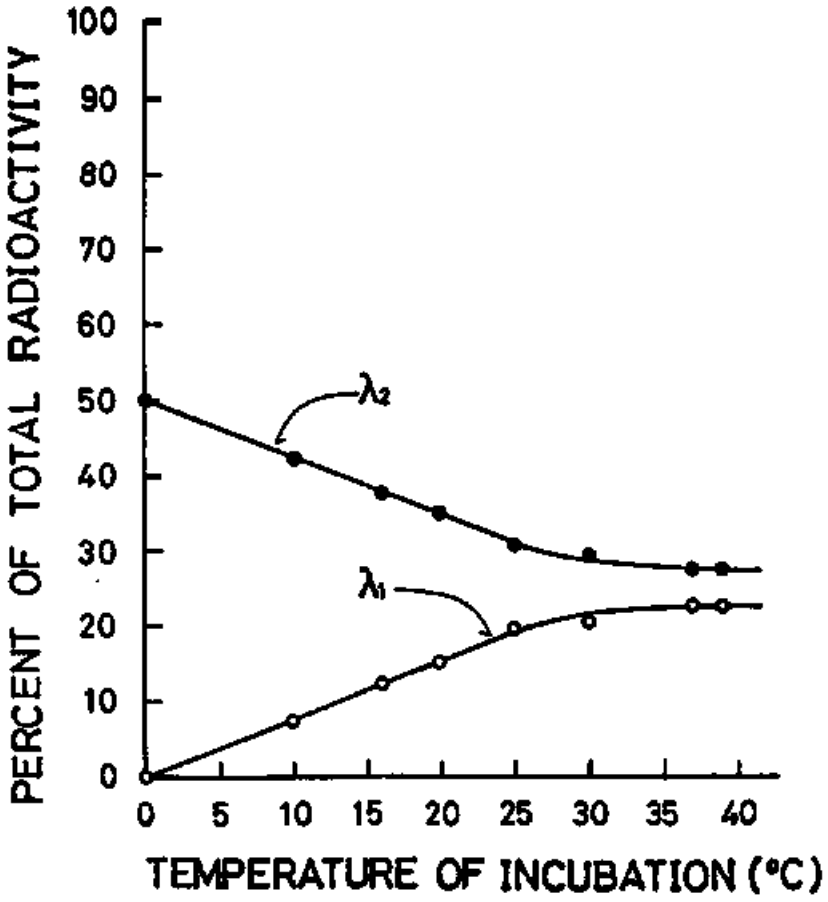

PERCENT OF TOTAL RADIOACTIVITY
100
90
80
70
60
50
40
30
20
10
0
λ2
λ1
0
5
10
15
20
25
30
35
40
TEMPERATURE OF INCUBATION (°C)

Fig. 7.

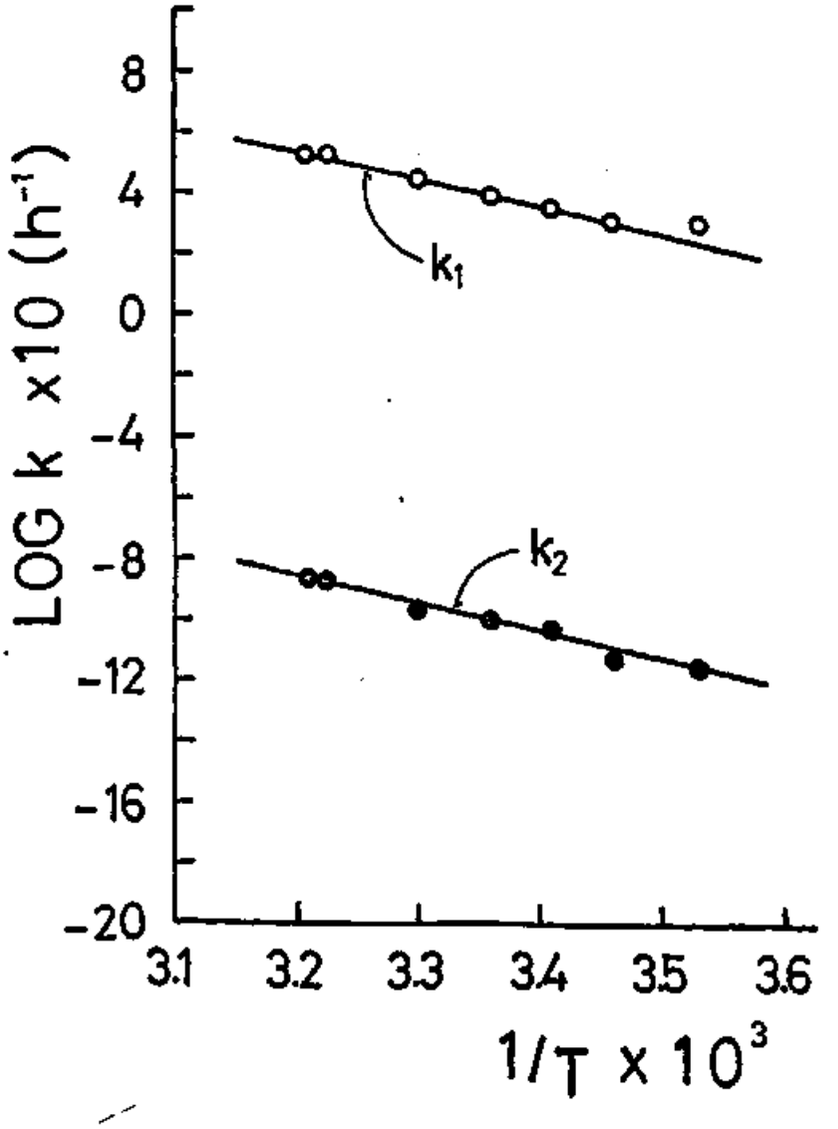


Fig. 8.

主論文 之.

EFFECTS OF INOSINE ON THE LEAKAGE OF PHOSPHATE IONS FROM THE GHOSTS OF RABBIT RED BLOOD CELLS

HIROSHI UTSUMI

Laboratory of Radiation Biology, Faculty of Science,
Kyoto University, Kyoto (Japan)

SUMMARY

Inosine caused reduction of the leakage of phosphate ions from rabbit red blood cell ghosts. The ghosts were prepared by reversible hypotonic hemolysis of red cells in the presence of adenosine triphosphate and magnesium ion, or magnesium ion only. Kinetic studies revealed that inorganic phosphate in ghosts was converted into organic phosphate during incubation with inosine, thus causing a decrease in the size of the inorganic phosphate pool which in turn reduced the leakage of phosphate ion into the medium. Metabolic inhibitors such as sodium fluoride and iodoacetate did not affect the reduction of phosphate leakage or the production of acid-soluble organic phosphates with inosine. The organic phosphates were separated and identified by paper chromatographic techniques. Among these, were ribose-5-phosphate, fructose-6-phosphate, phosphoglycerate, and adenosine tri- and di-phosphate.

INTRODUCTION

Nucleosides such as inosine and adenosine undergo extensive metabolism in red blood cells and their components. Dishe[1] observed that inosine, added to hemolysates, disappeared from hemolysates with simultaneous esterification of inorganic phosphate. Lionetti et al.[2-4] observed that red blood cell ghosts prepared by hypotonic hemolysis do not metabolize glucose, but convert inosine and inorganic phosphate into various phosphate esters. In the course of studies on phosphate release from red cell ghosts[5], we observed that phosphate ions in ghosts were divided into three fractions: acid-insoluble and acid-soluble organic, and inorganic phosphate; and that only the fraction of inorganic phosphate in the ghosts was involved in the leakage of phosphate. If the ghosts used in our previous work can metabolize inosine, it is expected that inosine influences the phosphate leakage by changing the phosphate distribution among the three phosphate fractions. The purpose of this communication is to report evidence that inosine metabolism affects phosphate release from ghosts.

MATERIALS AND METHODS

The procedures for preparing the three kinds of

ghosts and for measuring the ^{32}P-leakage were the same as those described in the previous paper[5]. Fresh rabbit blood was collected by venipuncture with heparin and centrifuged at 15,000xG for 5 minutes. After the plasma and buffy coat were sucked off, the packed red cells were treated as follows:

Preparation of "Mg-ghosts" and "ATP-Mg-ghosts": One volume of packed red cells was added to 10 volubes of ice cold hemolyzing solution (pH 7.5) containing carrier-free radioactive orthophosphate (12 μCi/ml ^{32}P, Radiochemical Centre, England) and either 4 mM $MgCl_2$ (Mg-ghosts) or 4 mM ATP-disodium salt and 4 mM $MgCl_2$ (ATP-Mg-ghosts). Within 2-3 minutes, 3 M NaCl solution was added to give a final concentration of about 150 mM. The hemolysate was incubated for 20 minutes at 37°C to insure the process of reconstitution[6,7]. The hemolysate was then centrifuged at 20,000xG at 0°C for 15 minutes and the reconstituted ghosts were washed 4 times with buffered salt solution consisting of 50 mM NaCl, 10 mM KCl and 90 mM tris-HCl (pH 7.5).

Preparation of "Common ghosts (C-ghosts)": One volume of packed cells was hemolyzed by 10 volumes of ice cold distilled water (pH 7.5) containing ^{32}P (12 μCi/ml). After standing for 20 minutes at room temperature, the hemolysate was centrifuged at at 20,000xG at 0°C for 15

minutes and the sedimented ghosts were washed 4 times by centrifugation with solution consisting of 9 parts of 12 mM $MgCl_2$ and 1 part of 17 mM tris-HCl (pH 7.5).

Time course experiments: After the final washing, one volume of the ^{32}P-loaded ghosts was resuspended in 30 volumes of ice cold buffered salt solution in the presence or absence of inosine. Unless otherwise stated, inosine was added to the incubation medium at a concentration of 5 mM. After removal of a sample at the start of incubation, parts of the suspensions were incubated for up to 3 hours, with constant shaking, in a water bath at 37° C. At appropriate intervals during during the incubation, 3 ml aliquots were withdrawn and immediately centrifuged at 20,000xG at 0° C for 15 minutes. One ml of the supernatant was pipetted into a stainless steel planchet. The test samples in the planchets were dried under an infrared lamp, and the radioactivity was measured with a GM-counter (ALOKA Co., Model TDC-2).

The amount of ^{32}P in the unit volume of the over-all suspension mixture was also measured. The total amount of ^{32}P retained in the ghosts was obtained as the ^{32}P count in the over-all suspension mixture minus that in the supernatant. The total amount of ^{32}P-leakage from the

ghosts during the incubation was obtained by subtracting the radioactivity in the supernatant at the start of incubation from the corresponding value found at a given incubation period.

The sedimented ghosts, as well as the supernatant, were treated with ice cold TCA [trichloroacetic acid; final concentration of 5 % (W/V)] and centrifuged. The radioactivity in an aliquot of the supernatant, i.e., the acid-soluble phosphate fraction, was measured according to the method described above. The inorganic phosphate (Pi) in the acid-soluble phosphate fraction was converted into phosphomolybdate and extracted with isobutanol-benzene (1:1) mixture according to a modification of the method of Martin and Doty[8], and the radioactivity of the extract was counted in the same manner. The amount of ^{32}P in the acid-soluble organic phosphate (designated as Po) was calculated as the difference between the amount of ^{32}P in the acid-soluble fraction and that in the Pi fraction. The amount of ^{32}P in the acid-insoluble phosphate fraction was calculated as the difference between the total amount of ^{32}P and that in the acid-soluble fraction. The amount of ^{32}P in various fractions was expressed as a percentage of the total amount of ^{32}P in

the ghosts at the start of incubation.

Hypotonic rehemolysis and temperature dependence experiments: The procedures were similar to those described in the previous paper[5].

Chromatographic experiments: The method for paper chromatographic analysis of the acid-soluble fraction in the ghosts was a modification of that of Schneider and Lepage[9]. The ghosts were treated with ice cold 10 % TCA and centrifuged. Barium acetate was added to the supernatant (acid-soluble fraction) after the pH was adjusted carefully to 8.2 with NaOH. Four volumes of cold ethanol were added and the precipitated esters were harvested by centrifugation after being maintained overnight at about 4° C. Barium ions were removed by swirling the precipitate in a beaker containing Amberlite-IR 120 (H^+) and then the clear supernatant after centrifugation was concentrated by lyophilization to give a final volume of 0.3 ml.

The concentrate was applied to filter paper (Toyo Roshi, # 50) with a micropipette and dried. The spots after drying were about 0.2 cm in diameter and gave approximately 10,000 counts per minute of ^{32}P when counted

by a GM-counter. The papers were developed by ascending chromatography with the solvent system: tertiary butanol, water, and picric acid [20:5:1 (V/V/W)]. After the papers had dried, autoradiographs of the chromatograms were made by placing the chromatograms in contact with X-ray films (Fuji No-Screen Type 200) for several days. The density of black spots of autoradiograms was measured with a densitometer (ATAGO Self-Recording Densitometer ATAGO Optical Works Co., Tokyo) to analyze quantitatively the increase and decrease of various phosphorylated intermediates during the course of incubation with inosine. The identification of the intermediates involved spot elution and co-chromatograpy with authentic compounds followed by radioautography and spraying with Hanes-Isherwood acidmolybdate reagent[10]. To obtain phosphorylated intermediates, commercial sodium or barium salts were converted to the free acids with Amberlite-IR 120 (H^+).

RESULTS

The effects of inosine upon ^{32}P-leakage from ghosts was investigated by time course experiments during

incubation with three kinds of ghosts (ATP-Mg-ghosts, Mg-ghosts and C-ghosts) of rabbit blood cells. Inosine caused considerable reduction of ^{32}P-leakage in ATP-Mg-ghosts, slight in Mg-ghosts, and little or none in C-ghosts. Therefore, the ATP-Mg-ghosts were hereinafter used in this study. The changes in distribution of ^{32}P in and outside ATP-Mg-ghosts during incubation with or without inosine are shown in Fig. 1. This shows that the addition of inosine to the incubation medium caused a significant decrease in ^{32}P counts leaking out from the ghosts (Fig. 1 A); these were derived from the acid-soluble fraction, since the acid-insoluble fraction remained relatively constant (Fig. 1 B). It also shows that the inosine rapidly increased in the amount of acid-soluble organic phosphate (^{32}Po) retained in the ghosts (Fig. 1 D). It is clear that inosine-induced decrease in phosphate leakage resulted from the decrease in cellular ortho-phosphate which was otherwise destined to leak out (Fig. 1 C).

This idea was also confirmed by hypotonic re-hemolysis experiment: At various intervals after incubation, aliquots of the ghost suspension were withdrawn and divided into two equal parts. One was used

for measuring the radioactivity in the acid-soluble fraction of ghosts. The other was rehemolyzed with distilled water (1:30) and the resultant hemolysate was centrifuged after about one hour. Per cent of ^{32}P released from ghosts by the hypotonic treatment was estimated from the radioactivity in the supernatant. The amount of unreleased acid-soluble ^{32}P by hypotonic treatment was obtained as the amount of acid-soluble ^{32}P retained in ghosts before the treatment minus that released from ghosts after the treatment. The ^{32}P released represents the leaky compartment of acid-soluble ^{32}P in ghosts[5]. Fig. 2 shows that the amount of unreleased acid-soluble ^{32}P by the treatment increased remarkably within first several minutes of incubation with inosine. In other words, the releasable acid-soluble ^{32}P decreased when inosine was present in the incubation medium.

The previous paper[5] showed that ^{32}P-leakage (y) from ghosts was represented by the following equation consisting of the fast and slow components:

$$y = \lambda_1(1-e^{-k_1 t}) + \lambda_2(1-e^{-k_2 t}) \quad \text{and} \quad \lambda_1+\lambda_2=\lambda \qquad (1)$$

where λ is the size of ^{32}P compartment in ghosts (given as a per cent of total activity in the ghosts at the start of incubation), k is the rate constant of ^{32}P-leakage from

ghosts, $\underline{t}$ is the incubation time at 37° C, $\underline{x}$ is the amount of acid-soluble ^{32}P in ghosts at the start of incubation. The subscripts 1 and 2 refer to the fast and the slow phase, respectively

The effects of inosine on the fast and slow leakage components were estimated from data of time course experiments as shown in Fig. 2 by the method described in the previous paper[5]: The data were plotted on semilog paper, each resultant curve was separated into two leakage components by the "backward projection" technique, and the numerical values in equation (1) were calculated. The mean values of $\underline{k}$ and $\underline{\lambda}$ from 9 experiments with inosine are shown in table I. The rate constant of the slow component ($\underline{k}_2$) was not influenced by the presence of inosine and the rate constant of the fast component ($\underline{k}_1$) increased only a little. On the other hand, the size of ^{32}P compartment of the fast component ($\underline{\lambda}_1$) decreased to about half by the presence of inosine. As a consequence, the size of the slow component ($\underline{\lambda}_2$) increased significantly according to the relation written in the equation ($\underline{\lambda}_1 + \underline{\lambda}_2 = \underline{x}$). This is accord with expectations based on the results of rehemolysis experiments (Fig. 2), since the fast component ($\underline{\lambda}_1$) corresponds to the fraction which was throughly released by the hypotonic treatment[5].

Fig. 3 shows the effects of varying concentration of inosine on ^{32}P-leakage in ATP-Mg-ghosts. The ^{32}P-leakage decreased no further above concentration of more than 5 mM, suggesting the saturation kinetics of enzymatic reaction.

To confirm the metabolic nature of the inosine-induced decrease in phosphate leakage, the time course of the ^{32}P-leakage from ghosts in the presence or absence of inosine was investigated at five different temperatures between 18°-37° C. The extents of ^{32}P-leakage induced by inosine at different temperatures were calculated as the difference in the amount of ^{32}P-leakage with and without inosine (Fig. 4 A). Rate constants for the inosine-induced reaction were calculated for the first 1.5 hours of incubation. Within this time, the reaction followed a first order kinetics: When the data in Fig. 4 A were replotted on semilog paper, each resultant curve gave a straight line, the slope of which give the rate constant ($\underline{k}$). The activation energy ($\underline{E}$) was calculated from the Arrhenius equation $d \ln \underline{k}/d(1/\underline{T}) = -\underline{E}/\underline{R}$ by plotting log $\underline{k}$ against $1/\underline{T}$, where the slope of the line is equal to $-0.219\ \underline{E}$ (Fig. 4 B)[5]. The activation energy found to be 20,000 calories per mole, further confirms the metabolic nature of the inosine-induced decrease in phosphate leakage[2].

The inosine-induced decrease in phosphate leakage was not influences by the presence of metabolic inhibitors; sodium fluoride (10^{-3}M NaF), iodoacetate (10^{-3}M IAA), and dinitrophenol (10^{-4}M DNP). On the other hand, inosine was able to be replaced by adenosine, guanosine and xanthosine, but not by cytidine, thymidine, ribose, glucose or sucrose. These results indicate that not glycolysis system but purine nucleoside phosphorylase have some connection with the inosine-induced decrease in phosphate leakage.

Fig. 5 shows the densitygraphs of the radioautographs obtained from paper chromatograms of phosphate compounds for different periods with inosine. At least 9 different compounds are resolved by this chromatographic system. At the begining of incubation, ^{32}P-counts were exclusively located at the position of inorganic phosphate (Pi, Rf 0.71). The amount of Pi decreased with increasing time of incubation at 37°C. On the contrary, ATP (Rf 0.08), ADP (Rf 0.14) and phosphoglycerate (FGA, Rf 0.56) increased up to 30 minute incubation, and then decreased slightly at 60 minutes. Ribose-5-phosphate (R-5-P, Rf 0.43), fructose-6-phosphate (F-6-P, Rf 0.47) and two other unknown organic phosphate (Rf's 0.28 and 0.34) continued to increase with incubation time. These results clearly show most of Pi in

the ghosts were converted into various forms of organic phosphate during incubation with inosine.

DISCUSSION

The results in the present paper demonstrate that inosine added to the incubation medium caused significant changes in the distribution of ^{32}P retained in the ghosts as well as that leaking out from them (Fig. 1); (1) decrease in ^{32}P-leakage from the ghosts, (2) decrease in inorganic ^{32}P inside the ghosts, and (3) increase in acid-soluble organic ^{32}P in the ghosts. The decrease in the amount of ^{32}P-leakage can be explained by the corresponding decrease in inorganic ^{32}P in the ghosts, since the latter phosphate is the sole source of leaking ^{32}P as found in the previous study[5]. It appears to be that the decrease in the amount of inorganic phosphate is a reflection of concomitant increase in that of acid-soluble organic phosphate in the ghosts. The conclusion reached is that inorganic phosphates in the ghosts were converted to organic phosphate during incubation with inosine, thus causing decreased size of inorganic phosphate pool inside the ghosts which in turn reduced the ^{32}P-leakage into the medium. Our model predicts that addition of inosine causes some changes in the size of ^{32}P compartment (λ_1 and λ_2) shown

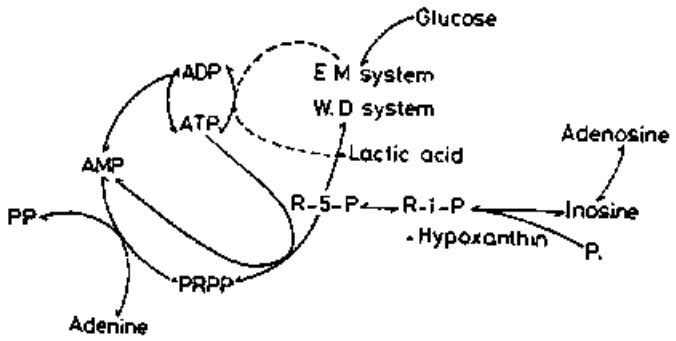
in equation (1), but does not affect the rate constants ($\underline{k}_1$ and $\underline{k}_2$) for ^{32}P-leakage. The results obtained (table I) agreed fairly well with our expection.

The results shown in Fig. 5 demonstrate that most of inorganic phosphate (Pi) present, at the begining of incubation, in TCA-soluble fraction in the ghosts were converted to various forms of organic phosphate during the incubation with inosine. The metabolic processes of phosphate related to inosine require several enzyme systems. The present study did not measure directly the activities of enzymes related to phosphate metabolism in the ghosts. However, these enzymes are very likely to exist in the ghosts, since the various phosphate esters found in the ghosts are those resulting from purine nucleoside phosphorylase activity. This enzyme is responsible for converting inosine and inorganic phosphate into the esters observed [11-14]. Several authors have reported the presence of the enzyme[11-14] in red blood cells and their ghosts in addition to phosphoribomutase[15], transaldolase[1,16], transketolase [1,16], phosphopentoisomerase[17,18] and epimerase[17,18] which could account for the synthesis of hexose phosphate and triose phosphate.

The activation energy was found to be 20,000 calories

per mole for the leakage of ^{32}P from the reconstituted ghosts incubated with inosine. The value is almost the same as that obtained for phosphate exchange in the ghosts by the presence of inosine[2]. This further confirms our notion that the inosine-induced decrease in ^{32}P-leakage is solely due to the reduction of leaky inorganic ^{32}P, by tranferring it enzymatically into organic phosphate in the presence of inosine.

Based on the present observations and those made by Nakao et al [19,20] and others[21,22], a schematic model for the phosphate metabolism which relates ^{32}P-leakage can be proposed. In the presence of inosine, cellular Pi is transferred to ribose moiety of inosine to produce R-1-P by phosphorolytic action of purine nucleoside phosphorylase and subsequently, R-5-P by means of phosphoribomutase. There are two possible pathways for the resultant R-5-P; ATP regeneration pathway proposed by Nakao et al[20], and pentose phosphate pathway (Warburg-Dickens system) in some manner linked to glycolysis (Emboden-Meyerhof system).



The results of the present study support the pentose phosphate pathway by the following reasons. The inosine-induced diminution of ^{32}P-leakage from ghosts was neither influenced by sodium fluoride nor iodoacetate which has been reported to inhibit ATP regeneration[19]. Furthermore, there found small ^{32}P activities at the positions of ATP and ADP on the radioautogram of acid-soluble fraction of ghosts incubated with inosine. On the other hand, intermediates of pentose phosphate pathway, F-6-P and PGA, were the major components on the radioautogram after 60 minute incubation (Fig. 5). This agrees with Lionetti's finding that the ghosts of human blood cells possessed the major components of pentose phosphate pathway[2-4,24].

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LEGENDS TO FIGURES

Fig. 1 The change in distribution of ^{32}P in and outside ATP-Mg-ghosts during incubation with or without inosine: The amount of ^{32}P in the acid-soluble organic phosphate (Po) was calculated as the difference between the amount of ^{32}P in the acid-soluble fraction and that in the Pi fraction. Each point is expressed in per cent of the total amount of ^{32}P in the ghosts at the start of incubation. Solid circles (with 5mM inosine), open circle (without inosine). See text for further explanation.

Fig. 2 The semilogarithmic plot of the per cent ^{32}P released from ATP-Mg-ghosts by hypotonic treatment: A; in the absence of inosine. B; in the presence of inosine. The curve (I) represents the amount of acid-soluble ^{32}P retained in ghosts before hypotonic treatment. The curves (III) and (II) represent the amount of acid-soluble ^{32}P un-released (×) and that of ^{32}P released (▲) from ghosts by the treatment, respectively. The curve (III) was calculated as the difference between the curve (I) and (II). The numbers on the abscissa indicates the time for which the ghosts were incubated before the treatment. See text for further explanation.

Fig. 3 The effect of varying concentration of inosine on the amount of ^{32}P-leakage after 60 minute incubation of ATP-Mg-ghosts.

Fig. 4 The effect of temperature on the inosine-induced decrease of ^{32}P-leakage from ATP-Mg-ghosts: The inosine-induced decrease of ^{32}P-leakage from ATP-Mg-ghosts was calculated as the difference between the amount of ^{32}P-leakage with and without inosine. The rate constant ($\underline{k}$) was calculated for the first 1.5 hours of incubation. Within this time, the inosine-induced decrease of leakage followed first order kinetics. (A); the time course of the inosine-induced decrease of ^{32}P-leakage. (B); the common logarithm of the rate constant ($\underline{k}$) expressed as function of the reciprocal of the absolute temperature ($\underline{T}$).

Fig. 5 The changes in the distribution of ^{32}P in acid-soluble phosphates obtained from ATP-Mg-ghosts during incubation with inosine: TCA-soluble phosphate fraction was precipitated as Ba-salt, and then applied on papers (Toyo Roshi # 50) after removing Ba ions by Amberlite-IR 120 (H^+). Ascending chromatography was carried out with a solvent system; tertiary butanol, picric acid and water (20:1:5, V/W/V) for 30 hours at

room temperature. The resultant chromatograms were placed in contact with X-ray films for several days to obtain autoradiograms. The density of black spots in the autoradiograms was measured by a densitometer, which is shown in the ordinate in relative values. The positions of various phosphate compounds are: ATP, Rf 0.08; ADP, Rf 0.14; R-5-P, Rf 0.43; F-6-P, Rf 0.47; FGA, Rf 0.56; Pi, Rf 0.71. The unidentified materials were also found at Rf's 0.00, 0.28, and 0.34. See text for further explanation.

TABLE I

The effect of inosine on the rate constant of ^{32}P-leakage (k) and the size of ^{32}P compartment (λ) of the equation (1).

	Fast phase components		Slow phase components	
Inosine	Absence (16)	Presence (9)	Absence (16)	Presence (9)
Rate constant ($hour^{-1}$)	k_1 3.407±0.236	k_1 4.295±0.289	k_2 0.134±0.007	k_2 0.135±0.001
Size of ^{32}P compartment (%)	λ_1 20.3±2.1	λ_1 10.5±1.3	λ_2 27.0±3.4	λ_2 36.1±7.0

The "backward projection" technique was employed to separate the semilog curve into two straight compartments, λ and k of resultant two components are expressed as the mean values with standard error. Numbers of determinations are shown in the parentheses. See text for further explanation.

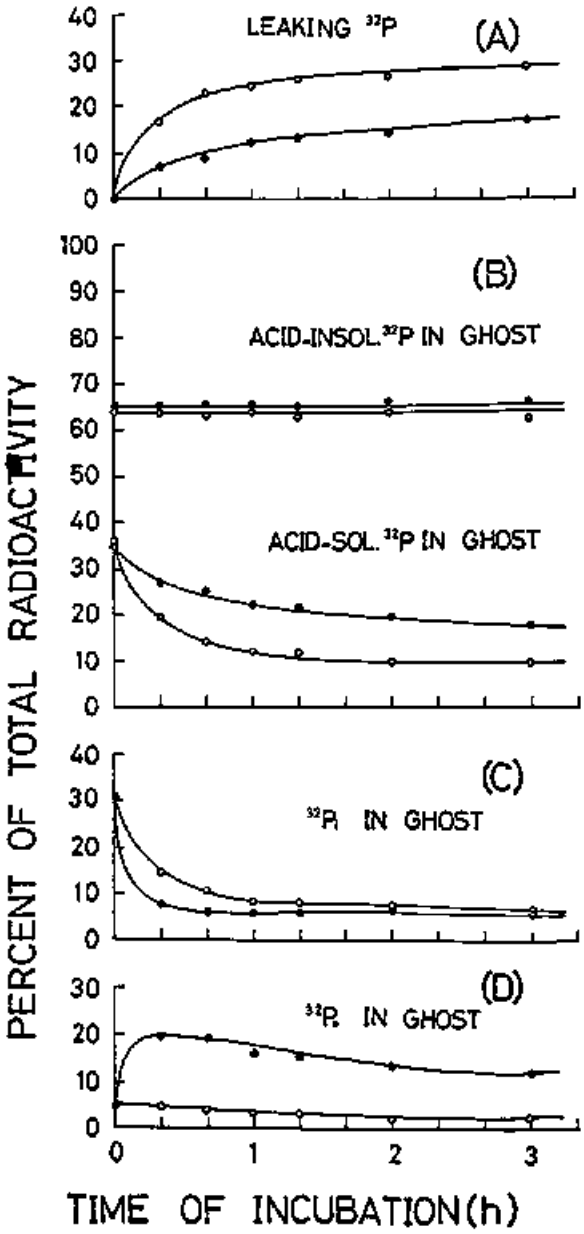
LEAKING ^{32}P
(A)
(B)
ACID-INSOL.^{32}P IN GHOST
ACID-SOL.^{32}P IN GHOST
(C)
^{32}P IN GHOST
(D)
^{32}P IN GHOST
PERCENT OF TOTAL RADIOACTIVITY
TIME OF INCUBATION(h)

Fig. 1.

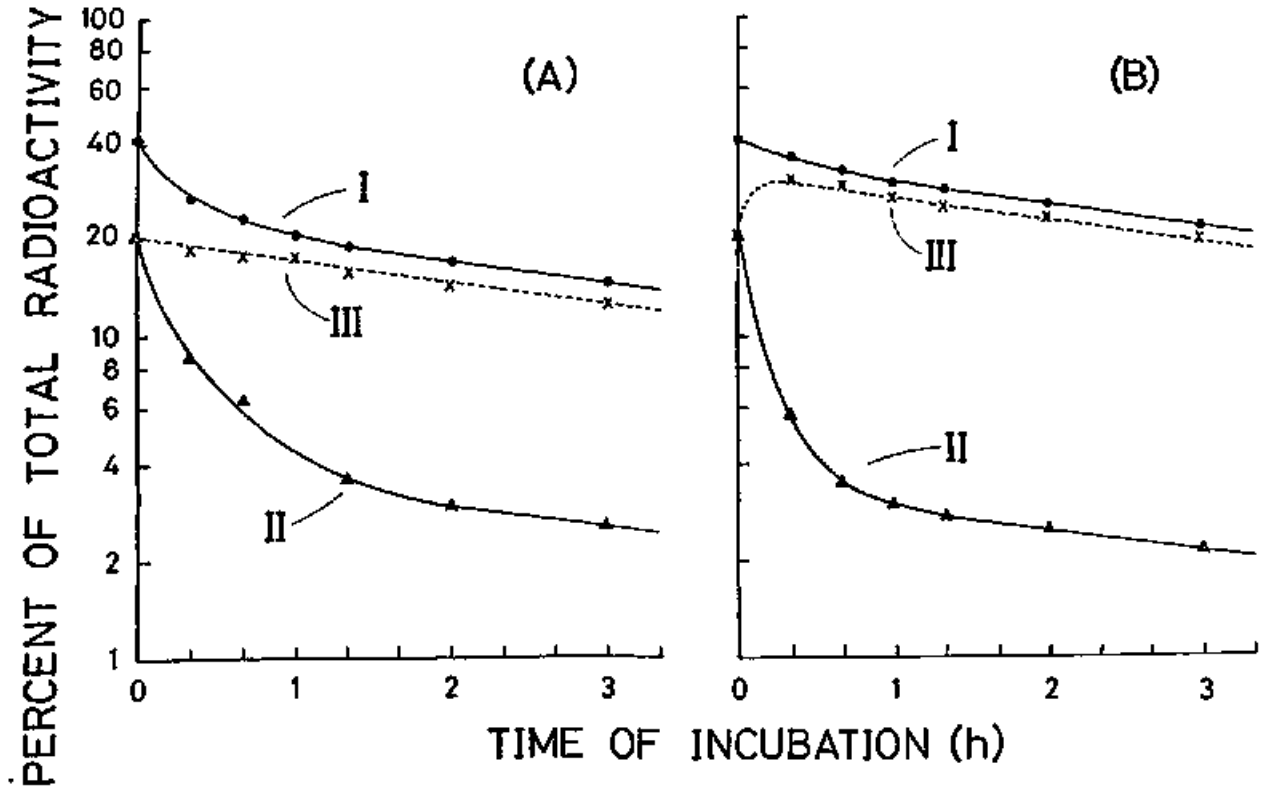
PERCENT OF TOTAL RADIOACTIVITY
100
80
60
40
20
10
8
6
4
2
1
(A)
(B)
I
II
III
0
1
2
3
TIME OF INCUBATION (h)

Fig. 2.

^{32}P-LEAKAGE FOR ONE HOUR (%)
40
35
30
25
20
15
10
5
0
0
5
10
15
20
CONCENTRATION OF INOSINE (mM)

Fig. 3.

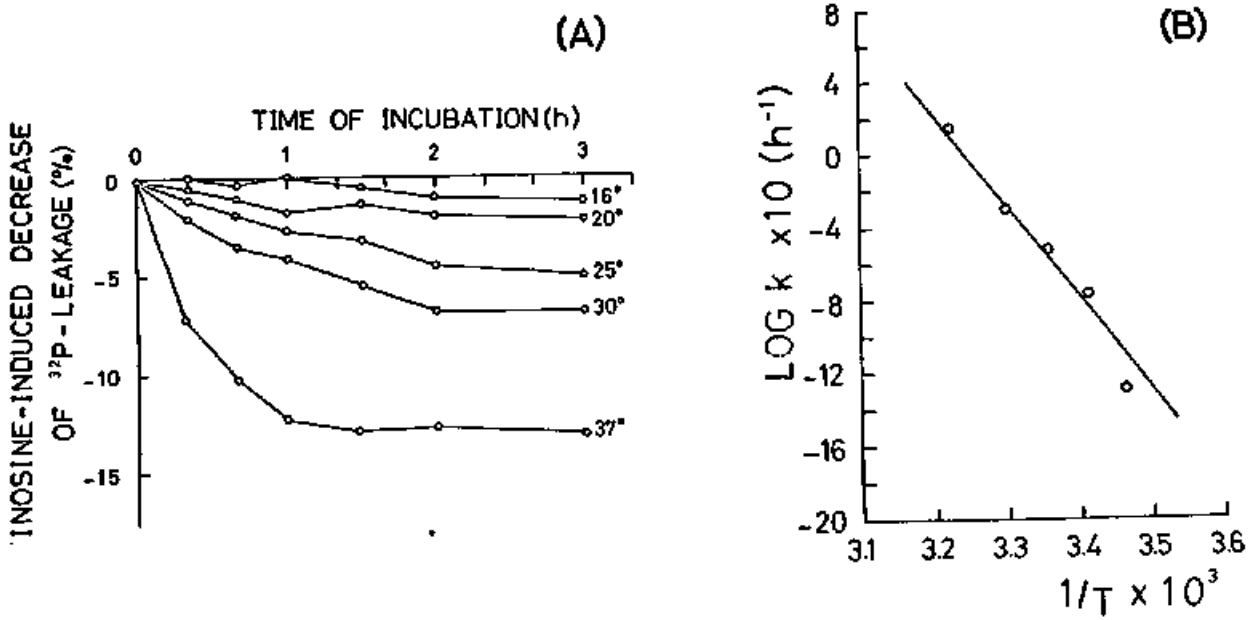
(A)
TIME OF INCUBATION(h)
0
1
2
3
0
-5
-10
-15
INOSINE-INDUCED DECREASE OF ^{32}P-LEAKAGE (%)
16°
20°
25°
30°
37°
(B)
8
4
0
-4
-8
-12
-16
-20
LOG k x10 (h^{-1})
3.1
3.2
3.3
3.4
3.5
3.6
1/T x 10^3

Fig. 4.

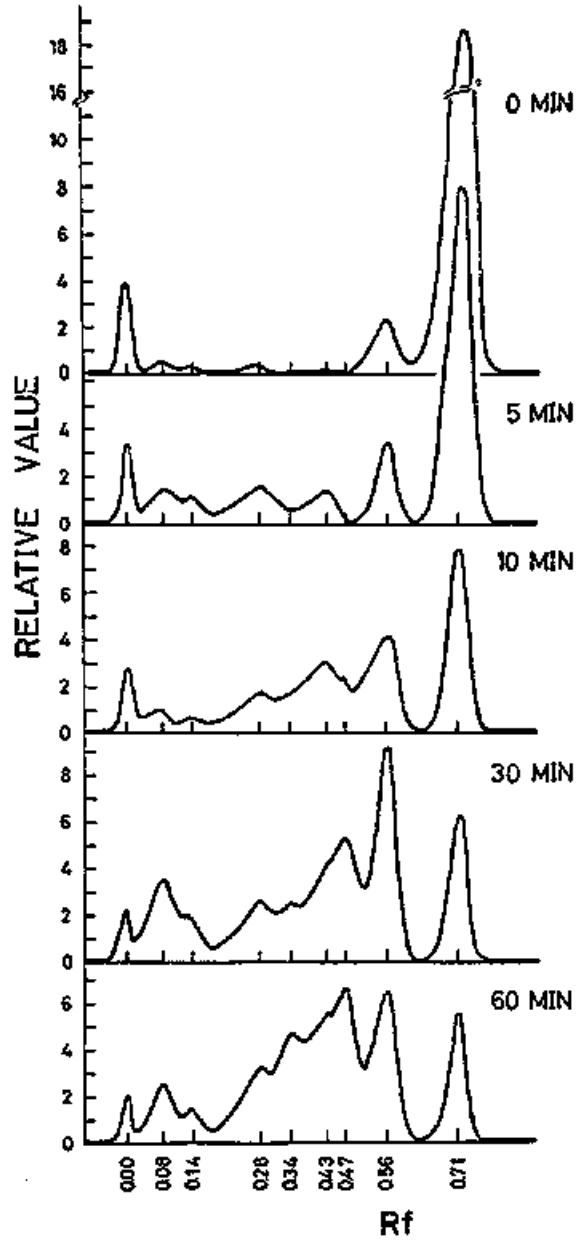
RELATIVE VALUE
0 MIN
5 MIN
10 MIN
30 MIN
60 MIN
0.00
0.08
0.14
0.28
0.34
0.43
0.47
0.56
0.71
Rf

Fig. 5.

主論文 3.

ROLE OF ADENOSINE TRIPHOSPHATE AND MAGNESIUM ION IN THE INOSINE-INDUCED REDUCTION OF PHOSPHATE-LEAKAGE FROM THE GHOSTS OF RABBIT RED BLOOD CELLS

HIROSHI UTSUMI

Laboratory of Radiation Biology, Faculty of Science,
Kyoto University, Kyoto (Japan)

SUMMARY

The retention of purine nucleoside phosphorylase was compared among three kinds of ghosts of rabbit red blood cells. Enzyme retention was highest in the ghosts prepared in the hemolyzing solution containing adenosine triphosphate (ATP) and magnesium ion (Mg), intermediate in the those prepared in the hemolyzing solution containing Mg, and lowest in those prepared in distilled water, suggesting that both ATP and Mg play important roles in preserving the enzyme. The role of ATP in preseeving the enzyme inside the ghosts could be substituted for nucleotide di- and tri-phosphates (adenosine diphosphate, uridine diphosphate, guanosine triphosphate, inosine triphosphate and cytidine triphosphate) or non-biological chelating agents (ethylene diamine-tetraacetate and glycolether diaminetetraacetate),

suggesting that ATP acts as a chelating agent rather than a common energy source.

INTRODUCTION

Since Hoffman[1] emphasized that ghosts have the advantage of considerable simplification in the study of membrane phenomena, numerous investigators[2-5] have studied membranes with red blood cell ghosts prepared by hypotonic hemolysis. However, as investigators have noted, the properties of the ghosts vary with the method of isolation [1-7]. The comparison of ghosts prepared by different methods is useful for the elucidation of many problems of membrane phenomena. In the previous paper[8], it was shown that inosine added to the incubation medium caused a considerable reduction of ^{32}P-leakage in ghosts prepared in a hemolyzing solution containing adenosine triphosphate (ATP) and magnesium ion (Mg). The reduction of ^{32}P-leakage caused by inosine was due to the decrease in pool size of cellular inorganic phosphate, resulting from conversion of inorganic (Pi) to organic phosphate (Po) by the action of purine nucleoside phosphorylase[9,10]. The present experiments were designed to clarify the role of ATP in

the reduction of ^{32}P-leakage by inosine. The results show that the effect of ATP is to retain efficiently purine nucleoside phosphorylase at the time of hemolysis.

MATERIALS AND METHODS

The procedures for preparing the three kinds of ghosts and for measuring the ^{32}P-leakage were the same as those described in the previous paper[8]. Fresh rabbit blood was collected by venipuncture with heparin and centrifuged at 15,000xG for 5 minutes. After the plasms and buffy coat were sucked off, the packed red cells were treated as follows:

Preparation of "Mg-ghosts" and "ATP-Mg-ghosts": One volume of packed red cells was added to 10 volumes of ice cold hemolyzing solution (pH 7.5) containing carrier-free radioactive orthophosphate (12 μCi/ml ^{32}P, Radiochemical Centre, England) and either 4 mM $MgCl_2$ (Mg-ghosts) or 4 mM ATP-disodium salt and 4 mM $MgCl_2$ (ATP-Mg-ghosts). Within 2-3 minutes, 3 M NaCl solution was added to give a final concentration of about 150 mM. The hemolysate was incubated for 20 minutes at 37° C to insure the process of reconstitution[1,2]. The hemolysate was then centrifuged at 0° C for 15 minutes, and the

reconstituted ghosts were washed 4 times with buffered salt solution consisting of 50 mM NaCl, 10 mM KCl and 90 mM tris-HCl (pH 7.5).

Preparation of "Common ghosts (C-ghosts)": One volume of packed cells was hemolyzed by 10 volumes of ice cold distilled water (pH 7.5) containing ^{32}P (12 μCi/ml). After standing for 20 minutes at room temperature, the hemolysate was centrifuged at 20,000xG at 0° C for 15 minutes and the sedimented ghosts were washed 4 times by centrifugation with a solution consisting of 9 parts of 12 mM $MgCl_2$ and 1 part of 17 mM tris-HCl (pH 7.5).

The ghosts used in the special experiments were prepared under the same conditions as the ATP-Mg-ghosts except for the substitution of various agents and divalent cations for ATP and Mg in the hemolyzing solution.

Time course experiments: After the final washing, one volume of the ^{32}P-loaded ghosts was resuspended in 30 volumes of ice cold buffered salt solution in the presence or absence of inosine (5 mM). After removal of a sample at the start of incubation, parts of the suspensions were incubater for up to 3 hours, with constant shaking, in a water bath at 37° C. At appropriate intervals during incubation, 3 ml aliquots were withdrawn and immediately

centrifuged at 20,000xG at 0° C for 15 minutes. One ml of the supernatant was pipetted into a stainless steel planchet. The test samples in the planchets were dried under an infrared lamp and the radioactivity was measured by a GM-counter (ALOKA Co., Model TDC-2).

The amount of ^{32}P in the unit volume of the over-all suspension mixture was also measured. The total amount of ^{32}P retained in ghosts was obtained as the ^{32}P count in the over-all suspension mixture minus that in the supernatant. Total amount of ^{32}P-leakage from ghosts during the incubation was obtained by subtracting the radioactivity in the supernatant at the start of incubation from the corresponding value found at given incubation period.

The sedimented ghosts, as well as the supernatant, were treated with ice cold TCA (trichloroacetic acid; final concentration of 5 % (W/V)) and centrifuged. The radioactivity in an aliquot of the supernatant, i.e., the acid-soluble phosphate fraction, was measured according to the same method as described above. The inorganic phosphate (Pi) in the acid-soluble phosphate fraction was converted into phosphomolybdate and extracted with isobutanol-benzene (1:1) mixture according to a modified method of Martin and Doty[11] and the radioactivity of the extract was

counted in the same manner. The amount of ^{32}P in the acid-soluble organic phosphate (designated as Po) was calculated as the difference between the amount of ^{32}P in the acid-soluble fraction and that in the Pi fraction. The amount of ^{32}P in the acid-insoluble phosphate fraction was calculated as the difference between the total amount of ^{32}P and that in the acid-soluble fraction. Amount of ^{32}P in various fractions was expressed in per cent of the total amount of ^{32}P in the ghosts at the start of incubation.

RESULTS

Table I shows the results of experiments to estimate the retention of purine nucleoside phosphorylase activity inside the three different kinds of ghosts after the first and second (rehemolysis of the ghosts obtained by the first hemolysis) hemolysis of rabbit red cells. In stead of directly measuring the phosphorylase activity preserved in the ghosts, we measured the enzyme activity released into the hemolyzing solution. It is seen in Table I that all the first and second hemolysate (ghost-free-hemolysate) of three kinds of ghosts (ATP-Mg-ghosts, Mg-ghosts and C-ghosts) had an ability to convert inorganic phosphate

(^{32}Pi) into acid-soluble organic phosphate (^{32}Po) when incubated with inosine. Only in the case of ATP-Mg-ghosts the second hemolysate was able to produce larger amounts of phosphorylated compounds from inosine than the first hemolysate. The second lysate of ATP-Mg-ghosts showed the largest amounts of phosphate conversion among three kinds of ghosts and the first lysate did the smallest. However, the sum of the percentages of inosine-stimulated conversion of Pi to Po obtained with first and second hemolysates are almost the same among three kinds of ghosts. The inosine-stimulated phosphate conversion is believed to be caused by the action of purine nucloside phosphorylase released at the time of hemolysis[9,10]. It may be concluded from these results that ATP-Mg-ghosts retained much of it after hemolysis but C-ghosts retained less. Though, the hemolysis seems to be complete in each ghosts, judging from the leakage of hemoglobin as shown in Table II.

To determine whether the enzyme retention in the ATP-Mg-ghosts is related to the presence of ATP and/or Mg in the ghosts, the leakage of ^{32}P with or without inosine was investigated in the ghosts lyzed in solutions containing various concentations of Mg and ATP. The results obtained are shown in Fig. 1. The ^{32}P-leakage from the

ghosts during 60 minute incubation was not affected by the presence of inosine if the concentration of ATP (or $MgCl_2$) was less than 1 mM in the hemolyzing solution containing 4 mM of $MgCl_2$ (or ATP). On the other hand, the ^{32}P-leakage was decreased by the presence of inosine when the ghosts were prepared by lyzing in the solution containing more than 2 mM of either one of ATP and Mg with 4 mM of the other. This indicates that the retention of the phosphorylase in ghosts after hemolysis was determined by the presence of both ATP and Mg.

The abilities of adenine derivatives to replace ATP were examined in the same manner with respect to the retention in the ghosts. Only adenosine diphosphate (ADP) was effective in revealing the inosine-induced reduction of ^{32}P-leakage. Adenine, adenosine and adenosine monophosphate were ineffective. Other nucleotide di-(uridine diphosphate; UDP) and tri-(guanosine triphosphate; GTP, uridine triphosphate; UTP, cytidine triphosphate; CTP, and inosine triphosphate; ITP) phosphate were also effective, but mono-(cytidine monophosphate; CMP, guanosine monophosphate; GMP, inosine monophosphate; IMP, and uridine monophosphate; UMP) phosphates were ineffective. All these nucleotide derivatives were used in a concentration of 4 mM.

An interesting observation was made as shown in Fig. 2; where it is seen that the ability of ATP to reveal the effect of inosine upon ^{32}P-leakage was completely stimulated by the addition of a chelating agent, ethylene diaminetetraacetate (EDTA), to the hemolyzing solution. Almost identical patterns of ^{32}P-distribution in and outside the ghosts were obtained with ATP-Mg-ghosts (Fig. 2 A) and EDTA-Mg-ghosts (Fig. 2 B). This is not surprising, since ATP itself has been known to act as a chelating agent[12-14]. Among other chelating agents tested, glycol ether diaminetetraacetate (GEDTA) was as effective as EDTA, but dimethyldithiocarbamate (DMDTC) and diethyldithiocarbamate (DEDTC) were not.

The substitute for Mg in the ATP-Mg-ghosts was searched in the same way. Only Ca was just as effective as Mg, and other divalent cations, Ba and Mn, were not effective.

DISCUSSION

The results shown in Table I can be interpreted as follows. The ghosts obtained after the first hemolysis still retained lots of purine nucleoside phosphorylase activity as judged from the ability of the ghosts-free second hemolysates to convert inorganic ^{32}P to organic

forms in the presence of inosine. The retention of the enzyme in the "first-cycle of ghosts" was the largest in ATP-Mg-ghosts, intermediate in Mg-ghosts and the smallest in C-ghosts. This indicates that both ATP and Mg are playing important roles in preserving the enzyme inside the ghosts. Indeed, more than 2 mM of either one of ATP and Mg together with 4 mM of the other were found to be necessary for the effective retention of the enzyme (Fig. 1). The enzyme retention was defined as the ability of ghosts to show the decrease in ^{32}P-leakage by the presence of inosine.

With regard to the mechanism by which ATP affects the phosphorylase retention, two alternatives are possible: (1) ATP is utilized as common energy source to maintain membrane structure of the ghosts[15-18], and (2) ATP acts as a chelating agent which is somewhat essential in cation binding by cellular membrane[19-22]. The first energy pathway was denied by the present observation that ATP in the ATP-Mg-ghosts can be replaced by nucleotide di- and tri-phosphates (ADP, UDP, CTP, ITP and GTP). On the other hand, the validity of the second alternative was clearly demonstrated by the finding that EDTA-Mg-ghosts showed almost identical behavior with ATP-Mg-ghosts in

regard to the leakage and retention of ^{32}P (Fig. 2). Another chelating agent, GEDTA, was also as effective as EDTA. These results suggests that ATP as well as nucleotide di-and tri-phosphates mentioned above act as a chelating agent to retain the enzyme phosphorylase in ghosts. Beta or gamma phosphate in these nucleotide derivatives may be associated with their chelating ability[12], since adenine, adenosine, and other nucleotide mono-phosphate were ineffective in the enzyme retention.

Effective substitution of Mg by Ca was also observed in making the ghost preparations retaining considerable amount of purine nucleoside phosphorylase activity. Thus, EDTA-Ca-ghosts were not distinguishable from ATP-Mg-ghosts with respect to inosine-induced reduction of ^{32}P-leakage (thereby, the phosphorylase retension in the ghosts). However, Mg in the ATP-Mg-ghosts could not be replaced by Ba or Mn. These results strongly suggest that Mg or Ca is essential for the enzyme retention in the ghosts, since both of these divalent cations are known to be necessary for maintaining membrane structures and functions from bacteria to mammals[19,24-26].

It is not known from the present study whether ATP and Mg act independently or synergistically in the retention

of the enzyme. However, it is well established that the effective substitutes for ATP found in the present experiment, i.e., ADP, EDTA, GEDTA all show strong affinity to form complex with Mg or Ca. Therefore, we prefer an co-ordinate action of ATP and Mg for the retention of the purine nucleoside phosphorylase in the ghosts.

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LEGENDS TO FIGURES

Fig. 1 Effect of varying concentration of ATP and Mg in hemolyzing solution on ^{32}P-leakage from rabbit red cell ghosts with and without inosine: The procedure for preparing the ghosts was the same as ATP-Mg-ghosts, except that rabbit red cells were hemolyzed in solutions containing various concentrations of Mg and ATP. The resultant ghosts were incubated in buffered salt solution with or without inosine (5 mM) at 37° C for one hour. The ^{32}P-leakage was estimated as the ^{32}P-activity in the supernatant after one hour incubation minus that found at the start of incubation. It was then converted to the per cent of the total amount of ^{32}P in the ghosts at the start of incubation.

Fig. 2 Changes in distribution of ^{32}P in and outside ATP-Mg-ghosts and EDTA-Mg-ghosts during incubation with or without inosine: (A) ATP-Mg-ghosts. (B) EDTA-Mg-ghosts; experimental conditions were the same as the ATP-Mg-ghosts, except for the replacement of ATP in hemolyzing solution with EDTA. The amount of ^{32}P in the acid-soluble organic phosphate (Po) was calculated as the difference between the amount of ^{32}P in the acid-soluble fraction and that in the Pi fraction. Every curve is expressed in per cent of the total amount of ^{32}P in ghosts

at the start of incubation (acid-insoluble ^{32}P + acid-soluble ^{32}P + leaking ^{32}P). Solid circles (with 5 mM inosine), open circle (without inosine). See text for further explanation.

Table I Conversion of ^{32}Pi into ^{32}Po in three different kinds of ghost-free-hemolysates incubated with inosine

	Conversion of ^{32}Pi into ^{32}Po (%)				Inosine-stimulated conversion (%)		
	1st hemolysate		2nd hemolysate		1st hemolysate	2nd hemolysate	1st + 2nd hemolysates
	Inosine	Control (Inosine-free)	Inosine	Control (Inosine-free)			
ATP-Mg-ghosts	66.8	(12.7)	83.9	(8.3)	54.1	75.6	129.7
Mg-ghosts	79.0	(9.3)	60.9	(10.0)	69.7	50.9	120.6
C-ghosts	96.5	(8.5)	49.3	(10.3)	88.0	39.0	127.0

The first hemolysate is ghost-free-hemolysate obtained after the ghosts prepared under the standard conditions were removed by centrifugation. The second hemolysate is ghost-free-hemolysate obtained after the resultant ghosts washed 4 times were also hemolyzed by distilled water (1:10) then centrifuged. The hemolysates ajusted to pH 7.5 were incubated in the presence of ^{32}Pi (about 12 Ci/ml) with and without 10 mM inosine at 37°C for 24 hours. The conversion of ^{32}Pi into ^{32}Po was expresred in the per cent of total radioactivity of the hemolysates. The inosine-stimulated conversion was calculated as the difference between ^{32}Po counts with and without inosine.

Table II. Leakage of hemoglobin at hemolysis

	First hemolysate (O. D.)
ATP-Mg-ghosts	0.0870
Mg-ghosts	0.0880
C-ghosts	0.0866

First hemolysate is the same sample as defined and used in Table I. O. D. is the optical density at 541 mμ.

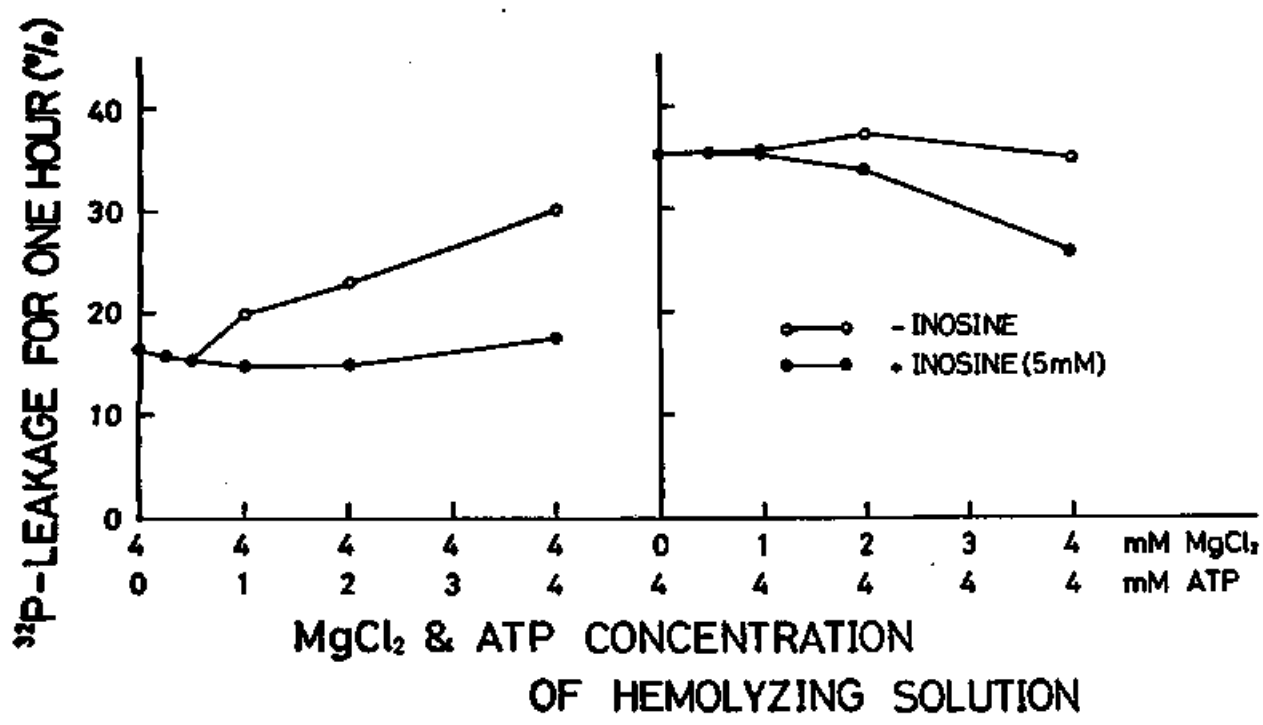
^{32}P-LEAKAGE FOR ONE HOUR (%)
40
30
20
10
0
- INOSINE
• INOSINE (5mM)
4 4 4 4 4 0 1 2 3 4 mM MgCl₂
0 1 2 3 4 4 4 4 4 4 mM ATP
MgCl₂ & ATP CONCENTRATION
OF HEMOLYZING SOLUTION

Fig. 1.

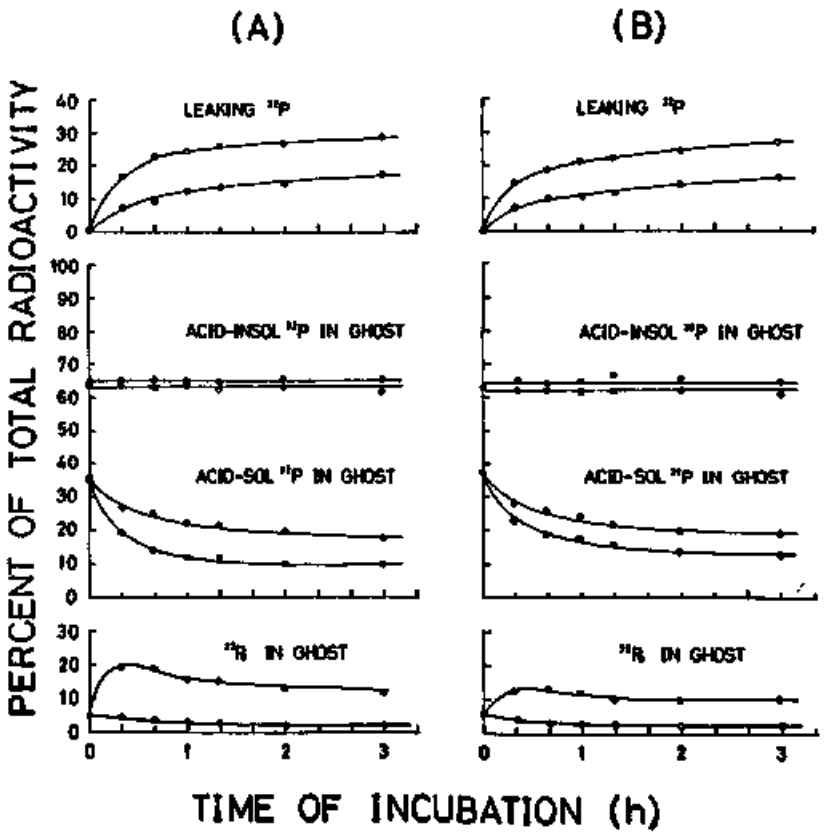
(A)
(B)
PERCENT OF TOTAL RADIOACTIVITY
LEAKING ³²P
ACID-INSOL ³²P IN GHOST
ACID-SOL ³²P IN GHOST
³²R IN GHOST
TIME OF INCUBATION (h)

Fig. 2.